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Respiratory Syncytial Virus: Virology, Reverse Genetics, and Pathogenesis of Disease

Peter L. Collins,

Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Building 50, Room 6517, 50 South Dr MSC 8007, Bethesda, MD 20892-8007, USA

Rachel Fearn, and

Department of Microbiology, Boston University School of Medicine, Boston, MA 02118, USA

Barney S. Graham

Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA

Peter L. Collins: pcollins@niaid.nih.gov

Abstract

Human respiratory syncytial virus (RSV) is an enveloped, nonsegmented negative-strand RNA virus of family *Paramyxoviridae*. RSV is the most complex member of the family in terms of the number of genes and proteins. It is also relatively divergent and distinct from the prototype members of the family. In the past 30 years, we have seen a tremendous increase in our understanding of the molecular biology of RSV based on a succession of advances involving molecular cloning, reverse genetics, and detailed studies of protein function and structure. Much remains to be learned. RSV disease is complex and variable, and the host and viral factors that determine tropism and disease are poorly understood. RSV is notable for a historic vaccine failure in the 1960s involving a formalin-inactivated vaccine that primed for enhanced disease in RSV naïve recipients. Live vaccine candidates have been shown to be free of this complication. However, development of subunit or other protein-based vaccines for pediatric use is hampered by the possibility of enhanced disease and the difficulty of reliably demonstrating its absence in preclinical studies.

1 Introduction

Respiratory syncytial virus (RSV) was first isolated in 1955, but its biochemical and molecular characterization remained rudimentary for many years due to its relatively inefficient growth in cell culture, pleomorphic and cell-associated nature, and physical instability. Detailed characterization began in 1981 with the molecular cloning and sequencing of RSV RNA. The resulting molecular tools have greatly increased our understanding of RSV and revolutionized research towards treatment and prevention. For example, candidate live-attenuated RSV vaccine viruses designed by reverse genetics are

presently in clinical studies (see chapters by H.Y. Chu and J.A. Englund, and by R.A. Karron et al., this volume). Vectored vaccines and recombinantly expressed viral antigen vaccines also are under development (see chapters by T.G. Morrison and E.E. Walsh, and by R.J. Loomis and P.R. Johnson, this volume). The murine monoclonal antibody that was the basis for palivizumab, used clinically for passive immunoprophylaxis in high-risk infants and children, was produced using recombinantly expressed antigen.

2 Classification

RSV is the type species of Genus *Pneumovirus*, Subfamily *Pneumovirinae*, Family *Paramyxoviridae*, Order *Mononegavirales*. Human RSV exists as two antigenic subgroups, A and B, that exhibit genome-wide sequence divergence (Table 1). The other members of this genus are bovine RSV (BRSV), ovine RSV(ORSV), and pneumonia virus of mice (PVM) (see Table 1 for amino acid sequence relationships). More pneumoviruses remain to be identified: recent wide-ranging fieldwork provided sequence evidence of RSV-like viruses in African bats (Drexler et al. 2012). Subfamily *Pneumovirinae* contains a second genus, *Metapneumovirus*, which consists of human and avian metapneumoviruses (HMPV and AMPV). The other subfamily of Family *Paramyxoviridae*, *Paramyxovirinae*, includes animal and human parainfluenza viruses (PIVs), mumps, measles, Nipah and Hendra viruses, and numerous other viruses whose number continues to expand (Drexler et al. 2012). Some of the notable features of RSV are summarized in Table 2.

3 Virion

The RSV virion consists of a nucleocapsid packaged in a lipid envelope derived from the host cell plasma membrane (Figs. 1, 2). Virions produced in cell culture consist of spherical particles of 100–350 nm in diameter and long filaments that usually predominate and are 60–200 nm in diameter and up to 10 µm in length (Jeffrey et al. 2003) (Fig. 1). In vitro, 95 % of progeny virus remains associated with the cell surface as particles that seemingly have failed to fully bud. In preparing virus stocks, infected cells typically are subjected to freeze-thawing, sonication, or vortexing to release attached virus, although this reduces infectivity and increases cellular contamination. RSV readily loses infectivity during handling and freeze-thawing due to particle instability and aggregation, although this can be partly overcome by excipients such as sucrose (Ausar et al. 2007). There is indirect evidence that the surface glycoproteins, especially F, are factors in the instability (Sastre et al. 2007; Rawling et al. 2011). The long filamentous shape of the particle likely also confers fragility.

The RSV envelope contains three viral transmembrane surface glycoproteins: the large glycoprotein G, the fusion protein F, and the small hydrophobic SH protein (Fig. 2). The nonglycosylated matrix M protein is present on the inner face of the envelope. The viral glycoproteins form separate homo-oligomers that appear as short (11–16 nm) surface spikes. RSV lacks neuraminidase or hemagglutinin activity and the F is known to be heavily sialylated, presumably because of the lack of a neuraminidase. There are four nucleocapsid/polymerase proteins: the nucleoprotein N, the phosphoprotein P, the transcription processivity factor M2-1, and the large polymerase subunit L (Fig. 2).

4 RNAs

The RSV genome (Fig. 3) is a single-stranded nonsegmented negative-sense RNA of 15,191–15,226 nt for six sequenced strains (subgroup A strain A2, 15,222 nt, GenBank accession number M74568, is the reference strain). RNA replication involves a complementary copy of the genome called the antigenome (Fig. 4). The genome and antigenome lack 5' caps or 3' polyA tails. The first 24–26 nt at the 3' ends of the genome and antigenome have 88 % sequence identity (Fig. 3b), representing conserved promoter elements that will be described later. The genome and antigenome are bound separately for their entire length by the N protein to form stable nucleocapsids. These are the templates for RNA synthesis and remain intact throughout the replicative cycle and in the virion. In addition, encapsidation protects the RNA from degradation and shields it from recognition by host cell pattern recognition receptors that initiate innate immune responses.

The genome has 10 genes in the order 3' NS1-NS2-N-P-M-SH-G-F-M2-L (Fig. 3). Each gene encodes a corresponding mRNA (Fig. 4) (Collins et al. 1986). The mRNAs have methylated 5' caps and 3' polyA tails. Each mRNA encodes a single major protein except for M2, which has two separate ORFs that overlap slightly and encode the M2-1 and M2-2 proteins. The downstream M2-2 ORF is accessed by ribosomes that exit the M2-1 ORF and reinitiate, a process that is influenced by upstream structure in the M2 mRNA (Gould and Easton 2007).

The 3' end of the genome consists of a 44-nt extragenic leader region that precedes the NS1 gene. The 5' end of the genome consists of a 155-nt extragenic trailer region that follows the L gene (Fig. 3). Each gene begins with a highly conserved 9-nt gene-start (GS) signal and terminates with a moderately conserved 12–14-nt gene-end (GE) signal that ends with 4–7 U residues (genome-sense) that encode the polyA tail by polymerase stuttering (Fig. 3b). The first nine genes are separated by intergenic regions that vary in length from 1 to 58 nt for the strains sequenced to date. These lack any conserved motifs, are poorly conserved between strains, and appear to be unimportant spacers, except that at some gene junctions the first nucleotide of the intergenic region is important for mRNA termination (Bukreyev et al. 2000; Harmon and Wertz 2002). A tolerance for intergenic variability is illustrated by the finding that incrementally increasing the length of an intergenic region in recombinant RSV up to 160 nt had little effect on gene expression or viral replication in vitro; however, this was moderately attenuating in mice, indicating that excessive length is restrictive (Bukreyev et al. 2000). The last two genes, M2 and L, overlap by 68 nt: specifically, the L GS signal is located 68 nt upstream of the end of the M2 gene (Collins et al. 1987) (Fig. 3b). The same overlap occurs in BRSV, and gene overlaps occur for some genes in some members of *Rhabdoviridae* and *Filoviridae*.

5 Proteins

RSV encodes 11 separate proteins, and thus is more complex than most members of *Paramyxovirinae*, which typically have 6–7 mRNAs encoding 7–9 separate proteins (Fig. 5). The N, P, M, F, and L proteins of RSV have clear orthologs throughout *Paramyxoviridae*, and their relative genome order is conserved (Fig. 5). Amino acid

sequence relatedness between RSV and *Paramyxovirinae* is low and is evident primarily for the F and L proteins and segments in the C-terminal region of N. The NS1, NS2, M2-1, and M2-2 proteins of RSV have no counterparts in *Paramyxovirinae*, and an SH protein (which is found in all members of *Pneumovirinae*) is present only in a few members of *Paramyxovirinae*.

The RSV F and G glycoproteins are the only viral neutralization antigens and are the major protective antigens. These proteins are reviewed elsewhere (see chapter by J.S. McLellan et al., this volume) and will only be briefly described here. The 574-amino acid F protein directs viral penetration and syncytium formation, like a typical F protein of *Paramyxoviridae*. The RSV F protein has general structural similarity to the F proteins of *Paramyxovirinae*, and similarly is synthesized as an inactive F0 precursor that is activated by cellular endoprotease to yield two disulfide-linked subunits, NH2-F2-F1-COOH. However, RSV F has two, rather than one, cleavage sites (Gonzalez-Reyes et al. 2001): one site (KKRKRR↓F-137) corresponds to that found in other paramyxoviruses, and the second site (RARR↓E-110) is located 27 amino acids upstream. RSV F0 is readily cleaved intracellularly by furin-like protease and is not a limiting factor for viral infectivity and tropism. The RSV F protein also binds to TLR-4, initiating signal transduction and innate immune responses (Haynes et al. 2001).

The 298-amino acid RSV large glycoprotein G, involved in attachment, appears to be unrelated to the *Paramyxovirinae* HN, H, or G attachment proteins. RSV G has a membrane anchor near its N-terminus, with the C-terminal two-thirds of the molecule being external. G also is produced as a secreted form—estimated to account for 80 % of released G protein (Hendricks et al. 1988)—that lacks the membrane anchor due to translational initiation at the second AUG in the ORF followed by proteolytic trimming. The ectodomain of G consists of two large divergent domains flanking a short central conserved segment. The divergent domains have a high frequency of amino acid differences among RSV strains; in addition, variants of G have been noted in nature containing partial intra-gene duplications (Eshaghi et al. 2012), small frame shifts, and C-terminal extensions. The central conserved region has a cysteine noose stabilized by two disulfide bonds, and this includes a CX3C motif. Surprisingly, this conserved region can be deleted with little effect on replication in vitro or in mice (Teng and Collins 2002). The large divergent domains have a high content of serine, threonine, and proline residues, as well as a high content of N-linked and, especially, O-linked sugars, and these large domains are thought to have extended, unfolded structures. These features also are characteristic of mucins, suggesting possible mucin mimicry by G, although the significance of this is unknown. The sugar side chains increase the estimated Mr of G from 32,000 for the polypeptide backbone to 80,000–90,000, and possibly 180,000 (Kwilas et al. 2009). The amino acid divergence and the presence of a sheath of host-specified sugars are thought to reduce immune recognition. Surprisingly, given its involvement in attachment, RSV lacking the G gene replicates in some cell lines as efficiently as wt RSV. A live-attenuated RSV vaccine candidate lacking most of the SH and G genes due to spontaneous deletions during passage in vitro appeared to be competent for replication in children, although it was highly restricted (Karron et al. 1997a). RSV isolates have been found, from infants exposed to or infected with human immunodeficiency virus,

with deletions spanning most of the G ectodomain, indicating that loss of most of G can occur in nature in some situations (Venter et al. 2011). Thus, G is a malleable, variable protein that is absolutely not essential for replication.

In addition to its role in attachment, G helps RSV evade host immunity. The region of G containing the CX3C motif noted above has been reported to mimic the CX3C chemokine fractalkine, with the effect of reducing the influx of immune cells into the lungs of RSV-infected mice (Tripp et al. 2001). The secreted form of G has been shown to interfere with antibody-mediated neutralization, acting as an antigen decoy as well as impeding cell-mediated neutralization of RSV by Fc receptor-bearing immune cells (Bukreyev et al. 2008). G has been speculated to mimic the receptor for tumor necrosis factor alpha (TNF- α), with the possible effect of inhibiting the antiviral effects of that cytokine (Langedijk et al. 1998). G can interact with DC-SIGN on human dendritic cells (DCs) and alter signaling pathways associated with antigen presentation (Johnson et al. 2012). Also, the central conserved domain of the G protein has been shown to inhibit the activation of several TLRs including TLR-4, thus countering the effect of the F protein (Polack et al. 2005).

The 64-amino acid SH protein is a transmembrane protein that is anchored near the *N*-terminus, with the *C*-terminus oriented extracellularly. Most of the SH protein is unglycosylated (*Mr* ~ 7,500), but SH also accumulates in a variety of forms from *Mr* 4,500 to up to 60,000 or more due to differences including N-linked sugar, polylactosaminoglycan, and translational initiation at the second methionine codon. This array of isoforms is conserved but their significance is unknown. SH forms pentameric pore-like structures that confer cation-selective channel-like activity (Carter et al. 2010; Gan et al. 2012), although the significance of this for RSV is not clear. Thus, the SH protein appears to be a viroporin, a class of small viral proteins that can modify membrane permeability and can affect budding and apoptosis. SH was reported to reduce apoptosis, but the effect was small (Fuentes et al. 2007). SH also appeared to inhibit signaling from TNF- α , an antiviral cytokine (Fuentes et al. 2007). Recombinant RSV lacking SH can replicate somewhat more efficiently *in vitro* than its wt parent—presumably due to its smaller genome size and smaller number of genes—and was slightly attenuated in mice and chimpanzees (Whitehead et al. 1999).

The 256-amino acid M protein plays key roles in virion morphogenesis. Early in infection, M is detected in the nucleus and may be responsible for the modest inhibition of host transcription during RSV infection, whereas later M is found associated with cytoplasmic viral inclusion bodies—thought to be the site of viral RNA synthesis—and the plasma membrane—the site of virion formation (Ghildyal et al. 2006). M appears to silence viral RNA synthesis by nucleocapsids, presumably in preparation for their packaging into virions (Ghildyal et al. 2006), and appears to be required for the transport of nucleocapsids from viral inclusion bodies to the plasma membrane (Mitra et al. 2012). M is not required to initiate the formation of viral filaments (thought to be the precursor to infectious virus), but in the absence of M the filaments remain stunted and immature (Mitra et al. 2012). Crystallography revealed an M protein monomer that is organized into compact *N*-terminal and *C*-terminal domains joined by a short linker (Money et al. 2009). The monomer surface contains a large positively charged area that extends across the two domains and may

mediate association with nucleocapsids and the negatively charged plasma membrane (Money et al. 2009).

The 391-amino acid N protein binds tightly to the genome and antigenome to form helical nucleocapsids, creating the templates for RNA synthesis. N protein expressed in bacteria bound to host RNA to form decamer rings that resembled one turn of the helical nucleocapsid (Tawar et al. 2009). Determination of the atomic structure of the N-RNA rings indicated that each N monomer consists of N-terminal and C-terminal domains separated by a hinge. Each N monomer was associated with seven nt of RNA, with the RNA groove at the hinge. Adjacent monomers were oriented in the same direction and loosely connected, providing flexibility that would allow polymerase access without disassembling the helix. Of the seven nt associated with each N monomer, nt 2–4 are oriented into the groove while the other four nt face outward. Passage of the polymerase may induce a transient hinge movement that makes the three buried nucleotides flip out to be accessible (Tawar et al. 2009). The N protein also has a role in antagonizing host innate immunity: N binds to the dsRNA-regulated protein kinase PKR and prevents it from phosphorylating eIF-2 α and inhibiting protein synthesis (Groskreutz et al. 2010).

The 241-amino acid P protein is an essential polymerase co-factor. It also acts as an adapter that binds to the N, M2-1, and L proteins to mediate interactions in the nucleocapsid/polymerase complex. In addition, P binds to free N protein monomers and delivers them to nascent genomes/antigenomes, thus preventing N from self-aggregating or binding to nonviral RNA (Castagne et al. 2004). The expression of N and P alone are sufficient to form viral inclusion bodies, which are large, dense cytoplasmic structures that are thought to be the sites of viral RNA synthesis. P exists as a homotetramer formed through a multimerization domain in the middle of the molecule, which is flanked by intrinsically disordered domains (Castagne et al. 2004; Llorente et al. 2006). The C-terminal region of P was shown to interact with the nucleocapsid by binding to a pocket on the surface of the N protein that includes discontinuous residues from positions 46–151 brought together in the folded structure (Galloux et al. 2012); other P-N binding sites may also exist, perhaps depending on conformation. P may contribute to conformational changes that help the polymerase access the RNA template (Castagne et al. 2004) and appears to be necessary for promoter clearance and chain elongation by the viral polymerase (Dupuy et al. 1999). It also appears to have a role in dissociating the M protein from the nucleocapsid during uncoating to initiate infection (Asenjo et al. 2008). P is the major phosphorylated RSV protein and contains phosphate at more than 10–12 sites, with different sites exhibiting differing rates of turnover due to interplay between cellular kinases and phosphatases (Asenjo et al. 2005). Many of the activities of P described above appear to be affected by dynamic phosphorylation/dephosphorylation at a subset of these sites, apparently involving a small percentage of the total phosphate content (Asenjo et al. 2006, 2008). Experiments in which P phosphorylation was reduced by mutational ablation or the use of inhibitor against cellular kinase supported the idea that phosphorylation is important for RSV replication, but that much of the low-turnover phosphate is not essential (Lu et al. 2002).

The 2,165-amino acid RSV L protein is very similar in length to its *Paramyxovirinae* counterparts and shares low but unambiguous sequence relatedness along nearly its entire

length. Specific segments are conserved within and beyond *Mononegavirales* that are thought to represent catalytic domains involved in polymerization. Analysis of RSV mutants has provided preliminary identification of functional regions in L, including the polymerization domain (Fix et al. 2011), a putative nucleotide-binding site involved in capping (Liuzzi et al. 2005) as well as residues that affect the efficiency of recognition of GE signals (Cartee et al. 2003).

The 194-amino acid M2-1 protein is an essential transcription processivity factor (Fearn and Collins 1999b; Collins et al. 1996, 1999). M2-1 accumulates in phosphorylated and nonphosphorylated forms and forms a homotetramer via an oligomerization domain at residues 32–63 (Tran et al. 2009; Cartee and Wertz 2001). The M2-1 protein binds RNA: the specificity of this interaction remains somewhat unclear, but M2-1 may preferentially bind RSV mRNAs (Cartee and Wertz 2001; Blondot et al. 2012). M2-1 also interacts with the P protein: binding to RNA or P involves partially overlapping domains in the center of the molecule (Blondot et al. 2012). M2-1 can be found in viral inclusion bodies and its presence there depends on interaction with P (Blondot et al. 2012). Interactions with RNA or the P protein are essential for the ability of M2-1 to support RNA synthesis and are competitive (Tran et al. 2009; Blondot et al. 2012), suggesting that P delivers M2-1 to the nucleocapsid and is then displaced. M2-1 also binds to the M protein and mediates its transport to inclusion bodies and interaction with nucleocapsids (Li et al. 2008). M2-1 contains a CCCH zinc finger motif near its *N*-terminus (residues 7–25) that is essential for its activity in viral RNA synthesis (Hardy and Wertz 2000). This unusual CCCH motif is also found in tandem in a family of cellular zinc finger proteins that includes tristetraprolin (TTP), which binds to AU-rich elements present in a number of host response mRNAs including cytokine mRNAs and affects their stability. Like TTP, M2-1 was recently shown to associate with cellular stress granules, which are involved in translational regulation under stress conditions, but the significance of this possible similarity is unclear (Fricke et al. 2013). M2-1 is unique to *Pneumovirinae*, although it shares structural homology with the VP30 transcriptional activator of *Filoviridae* (Blondot et al. 2012).

The M2-2 protein (88 or 90 amino acids, depending on the start site; Chang et al. 2005) is expressed at a low level in infected cells, and its status as a virion component is not known. Deletion of M2-2 from recombinant RSV results in a virus that exhibits delayed and reduced RNA replication and increased “runaway” transcription; this contrasts with wt RSV, for which transcription appears to be downregulated later in infection in favor of RNA replication (Bermingham and Collins 1999). These results suggest that M2-2 plays a role in regulating RNA synthesis; specifically, as the level of M2-2 increases during the time course of infection, it reduces transcription and promotes RNA. Consistent with a direct effect on RNA synthesis, over-expression of M2-2 inhibited RNA synthesis by mini-replicons and inhibited replication of complete RSV (Collins et al. 1996; Cheng et al. 2005). In addition, in experiments designed to produce virus-like particles, expression of M2-2 increased the efficiency of packing: this might reflect its effects on RNA synthesis or might be an unrelated activity (Teng and Collins 1998). Replication of M2-2 RSV in vitro is delayed but reaches titers comparable to wt RSV, whereas in mice and chimpanzees the virus was

restricted approximately 500- to 1000-fold compared to wt RSV (Bermingham and Collins 1999; Teng et al. 2000).

The NS1 and NS2 proteins (139 and 124 amino acids, respectively) are thought to be nonstructural. While the two proteins can function separately, they appear to form complexes and may have synergistic effects, but this is poorly understood (Spann et al. 2005; Swedan et al. 2011). NS1 and NS2 interfere with innate immune responses including interferon induction and signaling (Spann et al. 2005; Swedan et al. 2011). They also inhibit apoptosis, thereby prolonging the life of the cell and increasing viral yield (Bitko et al. 2007). In a mini-replicon system, co-expression of NS1—and, to a lesser extent, NS2—inhibited transcription and RNA replication, affecting both the genomic and antigenomic promoters (Atreya et al. 1998). These effects remain to be further investigated, but they suggest that NS1 and possibly NS2 might downregulate and restrain viral RNA synthesis. This may be comparable to effects shown for the C and V proteins of some members of *Paramyxovirinae* that, by downregulating viral RNA synthesis, avoids the accumulation of viral dsRNA that otherwise activates innate immunity. Recombinant RSV lacking the NS1 and/or NS2 genes have increased sensitivity to interferon, cause increased apoptosis, and replicate with reduced efficiency in cultured cells and experimental animals, with the effect of deleting NS1 being greater (Teng et al. 2000; Whitehead et al. 1999) (see chapter by S. Barik, this volume).

6 Transcription and RNA Replication

For transcription, the polymerase enters at the 3' end of the genome and copies the genes into their corresponding mRNAs (Fig. 4) by a sequential stop–start process guided by the GS and GE signals (Kuo et al. 1996). Synthesis of each mRNA initiates opposite the first nucleotide of the GS signal. Surprisingly, the initiating nucleotide can be selected by the polymerase independent of the template (Kuo et al. 1997). Thus, the GS signal triggers the start of mRNA synthesis; in addition, its complementary sequence present in the nascent transcript is thought to act as a signal for capping and cap methylation by the polymerase, based on analogy with other nonsegmented negative-strand RNA viruses (Wang et al. 2007). Capping and/or methylation appears to be essential for mRNA elongation: when RSV capping was blocked by a specific inhibitor, transcription produced uncapped abortive RNAs of ~ 45 to 50 nt (Liuzzi et al. 2005). Polymerase that is engaged in mRNA synthesis is unresponsive to encountering an additional GS signal, but encountering a GE signal triggers polyadenylation/termination of the mRNA and makes the traversing polymerase responsive to a GS signal (Kuo et al. 1996; Fearn and Collins 1999a). The triggering of polyadenylation/termination at the various GE signals is not completely efficient, and the polymerase occasionally continues synthesis through the next gene. This produces various readthrough mRNAs that account for approximately 10 % of total mRNA (Collins and Wertz 1983). RSV transcription has a polar gradient in which gene transcription decreases along the gene order (Fig. 4). This is typical for *Mononegavirales* and occurs because some of the transcribing polymerases disengage and exit the genome at the various gene junctions.

Studies with mini-replicons showed that N, P, and L are the viral proteins necessary for transcription, but under these conditions transcription terminates prematurely and

nonspecifically within several hundred nt, and genes that are further downstream are not significantly transcribed (Collins et al. 1995, 1996, 1999; Fearn and Collins 1999b). Fully processive transcription requires in addition the M2-1 protein, which can be present in relatively low relative molar amounts (Fearn and Collins 1999b; Collins et al. 1996). In mini-replicon experiments, M2-1 also decreased the efficiency of termination at the GE transcription signals—possibly a reflection of the same processivity activity—resulting in increased production of readthrough mRNAs (Hardy and Wertz 1998). These activities raise the possibility that M2-1 might affect the relative levels of expression of the various viral genes, such as by promoting sequential transcription and reducing the transcriptional gradient, but this has not been observed in infected cells (Fearn and Collins 1999b).

One-way sequential transcription does not provide for initiation at the L GS signal, since it is located upstream of the M2 GE signal, as noted (Fig. 3a). Studies with mini-replicons showed that, upon completing transcription of the M2-1 gene, the polymerase backtracks by retrograde scanning to initiate at the L GS signal (Fearn and Collins 1999a). Apart from the M2 GE and L GS signals, the overlap region did not appear to contain any other *cis*-acting elements needed for this activity. Furthermore, the polymerase was found to scan in both directions. This led to the realization that scanning may occur at each gene junction and may be the mechanism by which the next GS signal is located, and could explain the tolerance for intergenic variability noted above. The presence of the M2 GE signal within the L gene (due to the overlap) causes 90 % of newly initiated L gene transcripts to add polyA and terminate at the signal, producing a 68-nt polyadenylated RNA that does not appear to encode a protein and is not known to have any further significance (Collins et al. 1987). The synthesis of full-length L mRNA depends on the “error” of polymerase readthrough at the M2 GE signal (Collins et al. 1987). Whether the level of M2-1 protein affects this process remains to be evaluated. Although 90 % of L gene transcripts terminate at the M2 GE signal, there is not a steep drop in the transcriptional gradient at the L gene (Fearn and Collins 1999a; Kwilas et al. 2010). This indicates that backtracking is very active. The gene overlap appears to be an accidental arrangement that may provide no advantage but can be tolerated due to the scanning function of the polymerase.

The relative level of expression of the various RSV genes is determined mostly by the polar gradient of transcription. Thus, the most abundant mRNAs are for the NS1 and NS2 proteins that antagonize host responses. The expression of the L mRNA, the last gene in the order, is further reduced by an unidentified effect that appears to be post-transcriptional (Kwilas et al. 2010), possibly mRNA stability. Differences in the efficiency of polyadenylation/termination by the various GE signals due to natural sequence variation may affect the relative levels of gene expression by changing the amount of transcriptional readthrough (Harmon and Wertz 2002). On the one hand, increased readthrough spares the polymerase from disengagement at the gene junctions, providing more polymerase to downstream genes. On the other hand, ORFs that are at internal positions in readthrough transcripts are not efficiently translated, reducing the synthesis of proteins from genes downstream of inefficient GE signals. Thus, the overall effect is complex, and the impact of GE variation is not clear.

RSV RNA replication initiates opposite the first nucleotide at the 3' end of the genome. As with transcription, the initiating nucleotide can be selected independent of the template (Noton et al. 2010; Noton and Fearn 2011). The polymerase ignores the GS and GE signals and produces a full-length positive-sense replicative intermediate, the antigenome (Fig. 4). A fraction of antigenomes are modified at their 3' termini by addition of 1–3 nt, which are not copied into the genome RNA. The significance of the addition is not known, but it might represent a mechanism of promoter regulation (Noton et al. 2012). At least 10-fold more genome is produced than antigenome, both because the antigenome promoter is more efficient than that of the genome, and because synthesis from the genome is divided between RNA replication and transcription (Fearn et al. 2000; Hanley et al. 2010). Whereas efficient RNA replication by members of subfamily *Paramyxovirinae* requires that the genome nucleotide length be an even multiple of six ("rule of six"), RSV has no comparable length requirement. Studies with mini-replicons showed that N, P, and L are the viral proteins that are necessary and sufficient to direct RNA replication, and that RNA replication is unaffected by M2-1 (Collins et al. 1996; Grosfeld et al. 1995).

The *cis*-acting sequence elements at the 3' end of the genome that are involved in initiating transcription and RNA replication were analyzed using mini-replicons (Fig. 6). Transcription was found to require two sequence elements: the 3'-terminal 11 nt of the genome, and the presence of a nearby, downstream GS signal. In addition, the efficiency of transcription was increased by the presence of a U-rich sequence found at the end (nt 36–44) of the leader region (Fig. 6) (McGivern et al. 2005). Furthermore, the efficiency of transcription was affected by the length of the nucleotide chain between the 3'-terminal 11 nt and the GS signal: a length similar to that in wt RSV was the most efficient (McGivern et al. 2005; Fearn et al. 2000). For RNA replication, the 3'-terminal 11 nt of the genome was sufficient for initiation, but the resulting transcripts terminated prematurely and were unencapsidated: the synthesis of full-length encapsidated RNA depended on the additional presence of nt 16–34, implying the presence of an encapsidation signal within the first 34 nt of the genome (Fig. 6) (Cowton and Fearn 2005; McGivern et al. 2005). Thus, these studies identified the 3'-terminal 11 nt of the genome as a promoter element necessary for both transcription and RNA replication. Saturation mutagenesis showed that transcription was particularly dependent on positions 3, 5, 8, 9, 10, and 11. RNA replication depended on these same positions, as well as positions 1, 2, 6, and 7 (Fig. 6). The assignment at position four differentially affected transcription versus RNA replication: the wt assignment of 4G (negative-sense) favored transcription over RNA replication, whereas the substitution of 4C or 4U had the opposite effect (Fearn et al. 2002).

The *cis*-acting elements at the 3' end of the antigenome (i.e., containing the promoter involved in producing progeny genomes) are less well characterized. As noted, there is considerable sequence identity between the 3'-terminal 24–26 nt of the genome and antigenome, reflecting conserved promoter elements (Fig. 3b). In particular, the first 11 nt of the genome and antigenome differ only at position 4: the assignment in the genome (4G) is optimal for transcription, whereas that in the antigenome (4U) is optimal for RNA replication (Fearn et al. 2002). Mutation analysis of the antigenomic promoter showed that single nucleotide substitutions at positions 1–7 of this promoter have similar effects on RNA

replication as the corresponding mutations in the genome, although they do not behave identically (Peeples and Collins 2000; Noton and Fearn 2011). Mini-replicon studies showed that the first 36 nt of the antigenome were sufficient for initiation and synthesis of full-length genomes; however, production was increased by including nt 37–155 (Fearn et al. 2000). In infectious recombinant virus, nt 37–155 could be deleted with only minimal restriction of viral replication in vitro and in mice although, consistent with the mini-replicon studies, the synthesis of genomes was reduced (Hanley et al. 2010). In the same study, a mutant virus containing the complement of the 44-nt Le in place of the trailer region (i.e., in which the antigenomic promoter was replaced with the genomic promoter), replicated with reduced efficiency over multiple cycles and exhibited increased accumulation of stress granules (Hanley et al. 2010). One plausible interpretation of these data are that the 5' terminal 36 nt of the wt trailer RNA inhibits stress granule formation, which would otherwise reduce RSV replication, although another study has indicated that stress granules might benefit RSV replication (Lindquist et al. 2010).

The involvement of the 3'-terminal 11 nt of the genome in both transcription and RNA replication indicates that the first step in either process involves recognition of this segment by the polymerase. RNA replication initiates opposite the first nucleotide at the 3' end of the genome, as noted. For transcription, early events remain unclear. One possibility is that synthesis also initiates opposite the first position and produces a short transcript of the leader region. The polymerase may release this leader RNA—perhaps as abortive synthesis due to its uncapped nature—and reinitiate at the first GS signal. In an alternate model for transcription, the polymerase does not synthesize a leader RNA and instead scans without synthesis along the leader region to locate the first GS signal, similar to the proposed scanning at intergenic regions. These two models are discussed in detail by Cowton et al. (2006). It is not known whether a single form of polymerase complex is partitioned between transcription and RNA replication, or whether there are functionally distinct transcriptase and replicase complexes (which seems more likely). A common hypothesis for this type of virus is that there is a balance between transcription and RNA replication, and that the availability of soluble N protein promotes RNA replication at the expense of transcription, but this was not observed in studies with RSV mini-replicons (Fearn et al. 1997). The detailed mechanism of RNA synthesis, and how proteins such as P, M2-1, and M2-2 interact with the polymerase and nucleocapsid template, remains largely unknown. Cellular proteins likely are involved in RSV transcription and RNA replication. Soluble actin is important for RSV transcription, and its activity is augmented by profilin. Actin appears to function by binding directly to the viral N-RNA template and recruiting profilin to the complex (Harpen et al. 2009). Also, heat shock protein 70 associates with the RSV polymerase complex and there is evidence it augments RNA synthesis activity (Brown et al. 2005). A cell-free system for RSV RNA synthesis, in which polymerase activity is reconstituted with recombinant proteins and a synthetic template, has recently been developed and will provide the means for detailed characterization (Noton et al. 2012).

7 Summary of *cis*-Acting RNA Sequences

As detailed above, essential *cis*-acting signals include much of the 44-nt leader region, the 10 GS and GE signals, and the last ~ 36 nt of the trailer region (which encodes the 3' end of

the antigenome). These segments contain a total of ~ 300 nt, accounting for 2 % of the genome. It is likely that further fine-mapping will find that the essential positions in these segments comprise substantially fewer than 300 nt. As noted, there may be additional sequence in the M2 mRNA that promotes translation of the M2-2 ORF, and the efficiency of virus replication in vivo was increased slightly by including the complete trailer regions. There is presently no evidence of additional essential *cis*-acting signals.

8 Viral Replicative Cycle

RSV attachment and entry are mediated by the G and F glycoproteins, with no apparent contribution by SH, and are reviewed elsewhere (see chapter by J.S. McLellan et al., this volume). Entry occurs by fusion of the viral envelope with the cell plasma membrane. There is also evidence of entry by clathrin-mediated endocytosis, although endosomal acidification was not required and thus this pathway involves the same fusion mechanism as at the plasma membrane (Kolokoltsov et al. 2007; Srinivasakumar et al. 1991). Genome transcription and replication occur in the cytoplasm and the virus can grow in enucleated cells and in the presence of actinomycin D, indicating a lack of essential nuclear involvement.

By extrapolation from prototype members of *Mononegavirales*, incoming nucleocapsids engage in transcription by preformed polymerases (primary transcription). The availability of newly synthesized soluble viral N and P proteins promotes elongation of RNA replication products, leading to the production of full-length encapsidated antigenomes and genomes. Progeny genomes engage in transcription (secondary transcription) and RNA replication. Transcription and RNA replication occur concurrently. RSV mRNAs and proteins can be detected intracellularly at 4–6 h after infection and reach a peak accumulation by 15–20 h. At this time, transcription may be downregulated in favor of RNA replication and the production of genomes needed for packaging, and this appears to be mediated by the M2-2 protein (Bermingham and Collins 1999). However, there is no evidence of a change in the relative molar amounts of the various viral mRNAs during the infection time course (Fearn and Collins 1999b). The release of progeny virus begins by 10–12 h post infection, reaches a peak after 24 h, and continues until the cells deteriorate by 30–48 h.

As already noted, RSV-infected cells develop large cytoplasmic inclusion bodies that become evident by 12 h post-infection (Lindquist et al. 2011; Lifland et al. 2012). These have been shown to contain the viral N, P, M2-1, and L proteins, as well as viral RNA. As noted, the inclusion bodies are thought to be sites of RNA synthesis. More recently, the viral inclusion bodies have been shown to sequester key cellular signaling components and thereby inhibit cellular responses to infection. These include Mda5 and MAVS involved in interferon induction (Lifland et al. 2012) as well as p38 mitogen-activated protein kinase and O-linked N-acetylglucosamine transferase involved in stress responses and stress granule formation (Fricke et al. 2013). Infected cells develop filamentous surface projections that bear viral glycoproteins and may give rise to viral filaments (Fig. 1). Infected cell lines develop syncytia (Fig. 1) that are a major viral cytopathic effect and lead to the destruction of the monolayer, but that are much less evident in differentiated, polarized epithelium in vitro and in vivo (Johnson et al. 2007; Zhang et al. 2002).

RSV assembly and budding occur at the plasma membrane. In polarized cells, this occurs at the apical surface (Roberts et al. 1995; Zhang et al. 2002). These regions contain localized virus-modified lipid rafts involving all three viral surface proteins and the M protein (McDonald et al. 2004; Jeffree et al. 2003; McCurdy and Graham 2003; Henderson et al. 2002; Yeo et al. 2009). The minimum viral protein requirements for the formation of virus-like particles capable of delivering the viral genome to target cells are the F, M, N, and P proteins (Teng and Collins 1998), and expression of these proteins induced the formation of viral filaments (Utley et al. 2008). Both genome and antigenome have been detected in virions, suggesting a lack of selective packaging. Genome-containing nucleocapsids are much more abundant in the infected cell, and are correspondingly more abundant in virions. RSV appears to hijack cellular apical recycling endosomes for budding, a pathway that is distinct from that described for a number of other enveloped RNA viruses (Brock et al. 2003; Utley et al. 2008).

9 Spontaneous Mutations

RSV has a high rate of nucleotide substitution (10^{-3} to 10^{-4}), as is typical of RNA viruses. Virus passaged in vitro occasionally can acquire inserts of one (usually) or more U residues (negative sense) into the U tract at the end of GE signals or into untranslated sequence. Spontaneous deletion of the G and SH genes has been noted in vitro, and deletion of most of the G gene has been noted in vivo (Venter et al. 2011). Two different spontaneous intragenic duplications of a segment of the G gene have been documented in nature (Eshaghi et al. 2012), and in one case the resulting virus has spread worldwide and continued to evolve. The mechanism for this duplication is not known, but might involve backtracking of the polymerase following pausing at a secondary structure in the RNA. Recombination can occur when the polymerase jumps between templates during synthesis. This can create defective interfering genomes, which are typical for *Mononegavirales* but are poorly described for RSV. This also has the potential to create replication-competent mosaic genomes, although this appears to be very rare (Spann et al. 2003). Fresh clinical isolates of RSV may undergo some sort of adaptation to cell culture (Marsh et al. 2007), but this is poorly understood. In any event, passaged laboratory strains retain their virulence for chimpanzees and humans (Karron et al. 1997b; Whitehead et al. 1998). Circulating RSV appears to accumulate progressive changes in sequence and antigenicity, primarily in the G protein, in response to immune pressure, but this is a slow process occurring over decades.

10 Reverse Genetics

Reverse genetics involves the production of mini-replicons or complete virus from cDNA. Mini-replicons are versions of the genome or antigenome in which most or all of the viral genes are deleted and may be replaced by one or more convenient marker genes, such as chloramphenicol acetyl transferase or luciferase (Fig. 7a). The marker genes are each under the control of a set of RSV GS and GE signals. In the plasmid, the mini-replicon cDNA is flanked by a promoter for T7 RNA polymerase and a self-cleaving ribozyme sequence, which provide for synthesis of a negative- or positive-sense (depending on the orientation of the insert) mini-replicon with nearly correct ends. Typically, the mini-replicon plasmid is transfected into tissue culture cells together with so-called support plasmids that express

individual viral proteins under the control of T7 RNA polymerase promoters. T7 RNA polymerase can be supplied by a vaccinia virus recombinant, by a constitutively expressing cell line, or by a eukaryotic expression plasmid. One can provide whatever combination of RSV proteins is desired, and in the desired relative molar amounts. Depending on the support proteins and *cis*-acting elements included in the minigenome, this system can execute mini-replicon encapsidation, transcription, RNA replication, and particle morphogenesis (Fearn and Collins 1999a; Collins et al. 1996; Teng and Collins 1998; McGivern et al. 2005; Noton et al. 2010). Due to the small size and relative simplicity of the mini-replicons, this approach is ideal for detailed structure-function studies of *cis*-acting RNA signals or *trans*-acting viral proteins. Since the viral proteins are supplied *in trans*, the supply is independent of the mini-replicon, and thus the system can be used to study mutations that have drastic effects on mini-replicon transcription or RNA replication and could not be recovered and studied in complete recombinant virus.

The production of complete infectious virus from cDNA provides the means to introduce predetermined changes into infectious virus. The recovery of cDNA-derived virus involves co-transfection of cultured cells with a plasmid encoding a copy of the antigenome and support plasmids expressing proteins of the nucleocapsid/polymerase complex, namely N, P, M2-1, and L (Fig. 7b) (Collins et al. 1995, 1999). The expressed RSV antigenome and support proteins assemble into nucleocapsids that launch a productive infection. The expression of the positive-sense antigenome rather than the negative-sense genome avoids hybridization with the positive-sense RNAs expressed from the support plasmids. Recovery of infectious virus is not very efficient but, once recovered, cDNA-derived virus is readily propagated in the same manner as biologically derived virus and is distinguishable only by introduced mutations.

The genomes of RSV and other *Mononegavirales* have proven to be very amenable to manipulation. As noted, the RSV *cis*-acting RNA signals are short and circumscribed, and the modular organization of the genes also facilitates engineering: since the mRNAs and proteins are expressed independently of each other (apart from the transcription gradient), they can readily be manipulated independently. The upper limit for added sequence has not been determined for RSV and other members of *Mononegavirales*, and there probably is no strict packaging limit. The efficiency of replication *in vitro* decreases with added sequence, but the effect can be for inserts of several hundred or thousand nts minimal. For example, insertion of the 3.2-kb β -galactosidase gene into RSV (increasing its nucleotide length by 21 %) caused only a marginal reduction in replication *in vitro*. Effects *in vivo* of increasing genome length have not been studied carefully but appear to be substantially more restrictive

Reverse genetics provided new tools for basic virologic and pathogenesis studies. As already described, mini-replicon systems have been used to identify *trans*-acting proteins and *cis*-acting RNA signals and structures necessary for genome encapsidation, transcription, polymerase scanning, RNA replication, and virion morphogenesis. At the level of complete virus, mutants have been made that lack, for example, the NS1, NS2, SH, G, secreted G, and M2-2 genes, or which lack portions of these genes, or which have mutations to genome structures such as intergenic regions. In addition, RSV has been engineered to express fluorescent proteins, providing for real-time monitoring of infection.

Reverse genetics also provides the means to make new live-attenuated RSV vaccine candidates. This involves identifying mutations that attenuate RSV, and introducing attenuating mutations in desired combinations into RSV to create specific, well-characterized candidates for pre-clinical and clinical evaluation. As a second approach, PIVs have been developed as vectors to express the RSV F and G proteins. Vaccine candidates based on this technology are described elsewhere (see chapter by R.A. Karron et al., this volume).

All steps in the generation and development of RSV for evaluation in humans must be done with cells and reagents qualified for human product use. For example, this avoids components derived directly from animals (e.g., bacterial growth medium is plant-derived, porcine trypsin is replaced by recombinant trypsin). Recovery is performed in qualified cells, such as qualified lots of African green monkey Vero cells. T7 RNA polymerase is supplied by a co-transfected plasmid rather than by a recombinant virus or expressing cell line, and transfection is replaced by electroporation (Surman et al. 2007). These steps substantially reduce the efficiency of recovery. Of course, clinical trial material is subjected to extensive safety testing prior to use. Although engineered virus is of recombinant origin, it is propagated and “behaves” like biologic virus. The transfecting plasmids are needed only for the initial recovery, and the content of residual plasmid DNA quickly becomes insignificant upon passage of the virus. Producing virus from cDNA provides a virus with a short and well-defined passage history, which is important for safety and regulatory reasons (Surman et al. 2007). It thus has a safety advantage compared to biologically derived virus, which originates in an infected human and typically has been passaged extensively in cell culture. Cloned cDNAs also provide a stable vaccine seed. The ability to alter the virus provides a means to update a vaccine, for example, to modify the level of attenuation or to replace the F and G genes with those of newer strains.

The relatively poor growth and lability of RSV render some reverse genetics applications impractical. For example, RSV likely is not a good choice for obtaining high-level expression of foreign antigens from inserted genes. RSV has limited suitability as a human vaccine vector because most humans are exposed to RSV early in life and have substantial immunity, which would limit vector replication and immunogenicity.

11 Pathogenesis

RSV pathogenesis is complex and variable. Disease can range from mild to lethal and can encompass a wide range of acute upper and lower respiratory tract disease manifestations, from mild rhinitis at one extreme to bronchiolitis and pneumonia at the other. The heterogeneity of RSV disease also can include an association between severe disease in infancy and subsequent airway hyperreactivity during childhood and perhaps beyond, giant cell pneumonia in immunocompromised patients, and infection of the institutionalized elderly that result in exacerbation of underlying conditions and excess mortality. Host factors play a major role in disease heterogeneity: these include premature birth, young age or frail old age, low serum antibody titer, underlying conditions such as chronic lung or heart disease or immunosuppression, narrow or reactive airways, and other host factors of a more subtle genetic nature that remain to be fully described. More recently, the question of

heterogeneity in RSV strain virulence as another factor is being revisited. Many of these factors and unique host populations will be discussed in other chapters. Here we will briefly discuss the species and cell tropism of RSV, and the syndrome of vaccine-enhanced illness that occurred in children immunized with a formalin-inactivated RSV vaccine (FI-RSV) in the 1960s.

11.1 Viral Tropism

RSV infection and replication are largely restricted to the human. Although human RSV strains can infect other animals such as mice, cotton rats, sheep, and African green monkeys, these hosts are semi-permissive and transmission between animals or spread within a population does not occur. Conversely, the animal strains BRSV and PVM are strongly restricted in primates (Brock et al. 2012; Buchholz et al. 2000). Chimpanzees are the only animal host in which human RSV infects and replicates well enough to permit animal-to-animal transmission and to reliably produce respiratory tract disease.

In vivo, RSV is largely restricted to the superficial cells of the respiratory epithelium. RSV is recovered in abundance from nasal secretions, nasopharyngeal swabs, lung washes, and the sinuses. Using in situ hybridization and immunostaining for viral antigen, there is evidence in humans and cows naturally infected with their respective RSVs that epithelial cells from trachea, bronchi, and bronchioles are infected. Ciliated cells can clearly be shown to be infected in human airway epithelium, and it appears that basal epithelial cells are spared. Based on nearly circumferential staining of airway structures, it is possible that some nonciliated epithelial cells may also be infected, but this is not confirmed based on human pathology (Johnson et al. 2007). Both type I and type II alveolar pneumocytes are infected. Macrophages are sometimes shown to be immunostainpositive, but it is thought that this is most likely from phagocytized virus or virus proteins and not associated with replication. RSV RNA can be detected in the blood and in isolated reports from cerebrospinal fluid and the myocardium, and RSV antigens have been detected in circulating mononuclear leukocytes (Eisenhut 2006; Rohwedder et al. 1998), but these observations may not involve extrapulmonary infection. Infectious RSV is rarely recovered from an extrapulmonary location, and the few instances of recovery usually involve immunosuppressed individuals or experimental animals (Johnson et al. 1982; Eisenhut 2006).

Studies in vitro confirm a tropism for the superficial cells of the epithelium, but one that is not absolute. Infection of human airway epithelial cells in organ cultures is consistent with findings from human autopsy specimens and suggests RSV primarily infects ciliated cells (Zhang et al. 2002), although rare nonciliated cells may be infected in primary human airway epithelial cell cultures (Villenave et al. 2012) or adenoid organ cultures (Wright et al. 2005). Virus shedding occurs strictly at the apical membrane suggesting that polarized epithelium is the preferred cellular target. Paradoxically, studies with immortalized human cell in vitro have demonstrated that a wide variety of cell types can be infected. RSV can replicate in transformed cell lines derived from lung, kidney, liver, neural tissue, colon, breast, and ovarian tissues. Therefore, RSV replication is not necessarily restricted to its tissue of origin in the respiratory tract or confined to polarized epithelium, but highly differentiated cells (particularly ciliated cells that have undergone mesenchymal epithelial

transition and achieved planar polarity) are much more permissive than other cells in the context of airway epithelium. Intriguingly, in vitro cultures of primary human airway cells (which should be a more authentic substrate than cell lines) were refractory to RSV infection and replication when freshly seeded but gained susceptibility upon differentiation over a number of days, suggesting that infection of primary epithelial cells involves a differentiation-specific factor (Zhang et al. 2002). Non-epithelial human primary cells also have been infected with RSV, but generally with substantially less efficiency: these include fibroblasts, bone marrow stromal cells (Rezaee et al. 2011), eosinophils (Dyer et al. 2009), and DCs (Johnson et al. 2011). Myeloid and monocyte-derived DCs are more permissive (5–15 % infection rate) than plasmacytoid DCs (<1 %), and other nonadherent cells of hematopoietic origin are infected at very low frequencies. In addition, RSV can infect and replicate with reasonable efficiency in cell lines derived from other species, including African green monkeys, bovines, and hamsters.

These general observations suggest that there are cell-specific, species-specific, and context-specific factors that favor the growth of RSV in the human respiratory epithelium. Cellular tropism potentially can be determined by the presence of receptors (and co-receptors) for viral attachment and entry, host structures that may be utilized by the virus for various steps in the replicative cycle, restriction elements that may interfere with viral transcription or replication, or other innate defense mechanisms. Species-specific sequence differences in host molecules involved in virus replication may contribute to reduced efficiency of viral infection and replication in non-native hosts, since viruses have co-evolved with the native host. Reduced ability to counter antiviral responses—in particular the type I IFN response—in a non-native host also can be an important factor. The multiple mechanisms that RSV has evolved to block the host IFN response (see chapter by S.M. Varga and T.J. Braciale, this volume) illustrates the importance of this inhibition for the virus. For example, in mice, RSV replication is typically restricted almost exclusively to the type I alveolar pneumocytes, but a combined MAVS and MyD88 knockout that blocks IFN induction allows RSV to replicate in bronchiolar epithelium (Bhoj et al. 2008).

A specific receptor interaction that explains tissue tropism for RSV has not yet been identified. Both the RSV G and F glycoproteins can interact with heparan sulfate and are heavily glycosylated. Therefore, RSV can bind to proteoglycans and C-type lectins indiscriminately, which has complicated the search for a more specific RSV receptor. This may also contribute to the ability to infect a variety of cell types. Although the G glycoprotein is known as the attachment protein because of its strong glycosaminoglycan binding properties, G is not required for infection of cells in vitro, as noted, and thus the F glycoprotein may be more likely to have an essential, specific receptor-binding activity. Nucleolin has recently been described as a functional receptor for the RSV F protein, and it clearly increases permissivity to RSV infection (Tayyari et al. 2011). However, nucleolin is ubiquitously expressed and thus does not explain the strong preference that RSV has for respiratory epithelium. It is possible that there may be unique tissue-specific post-translational modifications of nucleolin, or a tissue-specific co-receptor, but these possible tissue-specific factors remain to be identified. Other interactions have been noted between RSV and cellular proteins, such as between F and TLR4 or G and CX3CR1, although possible contributions to attachment and tropism remain to be defined. There is very little

information available on cellular factors that may facilitate or restrict RSV replication and thereby affect cellular or species tropism, and this is an area that needs more attention.

11.2 Vaccine-Enhanced Illness

FI-RSV is an intramuscular, alum-adjuvanted, formalin-inactivated, whole-virus RSV vaccine that was evaluated in infants and young children in the 1960s. This vaccine was poorly protective and primed for enhanced disease upon RSV infection from subsequent natural exposure. The legacy of vaccine-enhanced illness has hovered over the field of RSV vaccine development for over 40 years, especially for the pediatric population. Much of the work on pathogenesis and animal model development for RSV has been devoted to understanding this phenomenon, because it was perceived to be the greatest barrier to having a robust pipeline of candidate vaccines for clinical evaluation. As a result, we have a better general understanding of the molecular mechanisms of RSV immunopathology and immunoregulation, and have developed better assays to measure the function and specificity of T cell and antibody responses. However, still there are uncertainties about the basis for the enhanced illness induced by FI-RSV and the ability to reliably determine preclinically whether new pediatric vaccine candidates are free of this phenomenon.

A series of clinical studies were done in the 1960s to evaluate the FI-RSV vaccine in children of different ages (Kim et al. 1969; Kapikian et al. 1969; Fulginiti et al. 1969; Chin et al. 1969). The vaccine did not protect against subsequent infection and, compared to control groups, caused a much greater frequency of severe disease, particularly in the youngest age group (<6 months of age). Of 31 infants immunized with FI-RSV, 25 required hospitalization following natural infection and two died (Kim et al. 1969). Only one of the 40 infants who received a control preparation of formalin-inactivated PIV3 required hospitalization. While the frequency of severe illness diminished as children became older (Fulginiti et al. 1969), enhanced disease was seen in children up to 37 months of age at the time of immunization (Fulginiti et al. 1969), and was common in children up to 23 months of age (Kapikian et al. 1969). The clinical manifestations appeared to be similar to those seen in the 3–4 % of infants in the general population who experience the most severe disease during RSV epidemics. This was suggestive of enhanced rather than altered disease, although this was not carefully evaluated. Lung pathology in two children who died showed peribronchiolar inflammation and fibrinous exudates composed of sloughed epithelial cells, mononuclear cells, neutrophils, and eosinophils causing obstruction of small airways. Eosinophilia among vaccine recipients was also noted in one study (Chin et al. 1969). This analysis was complicated by the occurrence of bacterial superinfection in both individuals. Although RSV-specific antibody responses measured by complement fixation were high, there was poor neutralizing and fusion-inhibiting activity (Murphy et al. 1986; Murphy and Walsh 1988). Delayed-type hypersensitivity (DTH) and significant lymphoproliferative responses were detected in peripheral blood mononuclear cells (Kim et al. 1976), similar to patients experiencing atypical measles syndrome which was a consequence of immunization with a whole, inactivated measles virus vaccine (Lennon et al. 1967). In addition, there was immunohistochemical evidence of immune complex deposition in small airways (Polack et al. 2002). Similar immune complex deposition has been noted in young adult patients with severe disease from pandemic influenza, with the presumption that cross-reactive low-

avidity antibody failed to clear virus and caused complement deposition and inflammation resulting in more severe disease (Monsalvo et al. 2011).

FI-RSV vaccine-enhanced illness has been modeled in mice (Graham et al. 1993), cotton rats (Connors et al. 1992), calves (Gershwin et al. 1998), and non-human primates (Kakuk et al. 1993; De Swart et al. 2002). Many of the pathological features of the disease have been recapitulated in these models, but because they are all either semi-permissive hosts or use an alternative virus and host (i.e., BRSV), these models likely are inexact surrogates for human infants. Unfortunately, the original phenomenon in the human vaccinees occurred long ago and was not well characterized. Thus, data from animal models cannot exclude the possibility that a vaccine may cause an enhanced illness syndrome in humans. However, vaccine-enhanced RSV disease appeared to be specific to RSV-naïve vaccinees, which also is the case in experimental animals. Studies in experimental animals indicate that enhanced disease is associated with protein-based RSV vaccines such as whole inactivated virus or purified proteins, but not with wild-type or attenuated replication-competent RSV. We know from clinical studies that replication-competent RSV given intranasally (Wright et al. 2007) or intramuscularly (Belshe et al. 1982) to human infants is not associated with enhanced RSV disease, nor is repeated exposure to RSV in nature associated with enhanced disease. Thus, replication-competent RSV vaccines are considered safe for testing in RSV-naïve infants, whereas protein-based vaccines are not.

Based on the diminished frequency of FI-RSV vaccine-enhanced illness in older children, vaccine studies in adults, and studies in animal models, it is thought that initial priming with live virus mitigates the effects of subsequent immunization with protein-based vaccines, and thus these vaccines are considered safe for use in older children and adults. We also know from animal models that the immunology of FI-RSV induced inflammation involves Th2-biased immune responses (Graham et al. 1993) including the production of IL-4, IL-5, and IL-13. Thus, enhanced disease likely involves altered immunologic priming, probably influenced by the mode of antigen presentation. Based on the available data from humans and animal models, two major immunological processes associated with the FI-RSV vaccine-enhanced disease can be articulated, and should be given careful consideration when deciding on the appropriate target population and regulatory requirements for new vaccine candidates. It is particularly important to consider these issues when the candidate vaccine is designed to be the first exposure to RSV antigen for RSV-naïve infants. First, RSV-specific antibody was induced with poor functional activity. This antibody was insufficient to restrict RSV replication, and may have contributed to immune complex deposition in small airways. Secondly, the FI-RSV enhanced disease was associated with a Th2-biased CD4 T-cell response characterized by cytokines related to allergic inflammation. Therefore, vaccines should be designed to optimize neutralizing activity and to avoid Th2-biased T-cell responses. There is evidence from epidemiological and genetic studies that associate immune components involved in allergic inflammation with severe RSV disease from primary infection. These data are discussed in other chapters in detail.

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References

- Asenjo A, Rodriguez L, Villanueva N. Determination of phosphorylated residues from human respiratory syncytial virus P protein that are dynamically dephosphorylated by cellular phosphatases: a possible role for serine 54. *J Gen Virol*. 2005; 86(Pt 4):1109–1120. [pii]. 10.1099/vir.0.80692-086/4/1109 [PubMed: 15784905]
- Asenjo A, Calvo E, Villanueva N. Phosphorylation of human respiratory syncytial virus P protein at threonine 108 controls its interaction with the M2-1 protein in the viral RNA polymerase complex. *J Gen Virol*. 2006; 87(Pt 12):3637–3642. [pii]. 10.1099/vir.0.82165-087/12/3637 [PubMed: 17098979]
- Asenjo A, Gonzalez-Armas JC, Villanueva N. Phosphorylation of human respiratory syncytial virus P protein at serine 54 regulates viral uncoating. *Virology*. 2008; 380(1):26–33. S0042-6822(08)00427-3 [pii]. 10.1016/j.virol.2008.06.045 [PubMed: 18706669]
- Atreya PL, Peeples ME, Collins PL. The NS1 protein of human respiratory syncytial virus is a potent inhibitor of minigenome transcription and RNA replication. *J Virol*. 1998; 72(2):1452–1461. [PubMed: 9445048]
- Ausar SF, Espina M, Brock J, Thyagarayapuran N, Repetto R, Khandke L, Middaugh CR. High-throughput screening of stabilizers for respiratory syncytial virus: identification of stabilizers and their effects on the conformational thermostability of viral particles. *Hum Vaccin*. 2007; 3(3):94–103. [pii]. 10.1099/vir.0.82165-04149 [PubMed: 17426457]
- Belshe RB, Van Voris LP, Mufson MA. Parenteral administration of live respiratory syncytial virus vaccine: results of a field trial. *J Infect Dis*. 1982; 145(3):311–319. [PubMed: 7037983]
- Bermingham A, Collins PL. The M2-2 protein of human respiratory syncytial virus is a regulatory factor involved in the balance between RNA replication and transcription. *Proc Natl Acad Sci U S A*. 1999; 96(20):11259–11264. [PubMed: 10500164]
- Bhoj VG, Sun Q, Bhoj EJ, Somers C, Chen X, Torres JP, Mejias A, Gomez AM, Jafri H, Ramilo O, Chen ZJ. MAVS and MyD88 are essential for innate immunity but not cytotoxic T lymphocyte response against respiratory syncytial virus. *Proc Natl Acad Sci U S A*. 2008; 105(37):14046–14051. 10.1073/pnas.0804717105 [PubMed: 18780793]
- Bitko V, Shulyayeva O, Mazumder B, Musiyenko A, Ramaswamy M, Look DC, Barik S. Nonstructural proteins of respiratory syncytial virus suppress premature apoptosis by an NF-kappaB-dependent, interferon-independent mechanism and facilitate virus growth. *J Virol*. 2007; 81(4):1786–1795. JVI.01420-06 [pii]. 10.1128/JVI.01420-06 [PubMed: 17151097]
- Blondot ML, Dubosclard V, Fix J, Lassoued S, Aumont-Nicaise M, Bontems F, Eleouet JF, Sizun C. Structure and functional analysis of the RNA- and viral phosphoprotein-binding domain of respiratory syncytial virus M2-1 protein. *PLoS Pathog*. 2012; 8(5):e1002734. 10.1371/journal.ppat.1002734 [PubMed: 22675274]
- Brock SC, Goldenring JR, Crowe JE Jr. Apical recycling systems regulate directional budding of respiratory syncytial virus from polarized epithelial cells. *Proc Natl Acad Sci U S A*. 2003; 100(25):15143–15148. [PubMed: 14630951]
- Brock LG, Karron RA, Kreml CD, Collins PL, Buchholz UJ. Evaluation of pneumonia virus of mice as a possible human pathogen. *J Virol*. 2012; 86(10):5829–5843. 10.1128/JVI.00163-12 [PubMed: 22438539]
- Brown G, Rixon HW, Steel J, McDonald TP, Pitt AR, Graham S, Sugrue RJ. Evidence for an association between heat shock protein 70 and the respiratory syncytial virus polymerase complex within lipid-raft membranes during virus infection. *Virology*. 2005; 338(1):69–80. 10.1016/j.virol.2005.05.004 [PubMed: 15936795]
- Buchholz UJ, Granzow H, Schuldt K, Whitehead SS, Murphy BR, Collins PL. Chimeric bovine respiratory syncytial virus with glycoprotein gene substitutions from human respiratory syncytial

- virus (HRSV): Effects on host range and evaluation as a live-attenuated HRSV vaccine. *J Virol.* 2000; 74(3):1187–1199. [PubMed: 10627529]
- Bukreyev A, Murphy BR, Collins PL. Respiratory syncytial virus can tolerate an intergenic sequence of at least 160 nucleotides with little effect on transcription or replication in vitro and in vivo. *J Virol.* 2000; 74(23):11017–11026. [PubMed: 11069997]
- Bukreyev A, Yang L, Fricke J, Cheng L, Ward JM, Murphy BR, Collins PL. The secreted form of respiratory syncytial virus G glycoprotein helps the virus evade antibody-mediated restriction of replication by acting as an antigen decoy and through effects on Fc receptor-bearing leukocytes. *J Virol.* 2008; 82(24):12191–12204. JVI.01604-08 [pii]. 10.1128/JVI.01604-08 [PubMed: 18842713]
- Cartee TL, Wertz GW. Respiratory syncytial virus M2-1 protein requires phosphorylation for efficient function and binds viral RNA during infection. *J Virol.* 2001; 75(24):12188–12197.10.1128/JVI.75.24.12188-12197.2001 [PubMed: 11711610]
- Cartee TL, Megaw AG, Oomens AG, Wertz GW. Identification of a single amino acid change in the human respiratory syncytial virus L protein that affects transcriptional termination. *J Virol.* 2003; 77(13):7352–7360. [PubMed: 12805433]
- Carter SD, Dent KC, Atkins E, Foster TL, Verow M, Gorny P, Harris M, Hiscox JA, Ranson NA, Griffin S, Barr JN. Direct visualization of the small hydrophobic protein of human respiratory syncytial virus reveals the structural basis for membrane permeability. *FEBS Lett.* 2010; 584(13):2786–2790. S0014-5793(10)00395-9 [pii]. 10.1016/j.febslet.2010.05.006 [PubMed: 20471980]
- Castagne N, Barbier A, Bernard J, Rezaei H, Huet JC, Henry C, Da Costa B, Eleouet JF. Biochemical characterization of the respiratory syncytial virus P-P and P-N protein complexes and localization of the P protein oligomerization domain. *J Gen Virol.* 2004; 85(Pt 6):1643–1653. [PubMed: 15166449]
- Cheng X, Park H, Zhou H, Jin H. Overexpression of the M2-2 protein of respiratory syncytial virus inhibits viral replication. *J Virol.* 2005; 79(22):13943–13952. 79/22/13943 [pii]. 10.1128/JVI.79.22.13943-13952.2005 [PubMed: 16254330]
- Chin J, Magoffin RL, Shearer LA, Schieble JH, Lennette EH. Field evaluation of a respiratory syncytial virus vaccine and a trivalent parainfluenza virus vaccine in a pediatric population. *Am J Epidemiol.* 1969; 89(4):449–463. [PubMed: 4305200]
- Collins PL, Wertz GW. cDNA cloning and transcriptional mapping of nine polyadenylated RNAs encoded by the genome of human respiratory syncytial virus. *Proc Natl Acad Sci U S A.* 1983; 80(11):3208–3212. [PubMed: 6190173]
- Collins PL, Dickens LE, Buckler-White A, Olmsted RA, Spriggs MK, Camargo E, Coelingh KV. Nucleotide sequences for the gene junctions of human respiratory syncytial virus reveal distinctive features of intergenic structure and gene order. *Proc Natl Acad Sci U S A.* 1986; 83(13):4594–4598. [PubMed: 3460060]
- Collins PL, Olmsted RA, Spriggs MK, Johnson PR, Buckler-White AJ. Gene overlap and site-specific attenuation of transcription of the viral polymerase L gene of human respiratory syncytial virus. *Proc Natl Acad Sci U S A.* 1987; 84(15):5134–5138. [PubMed: 2440043]
- Collins PL, Hill MG, Camargo E, Grosfeld H, Chanock RM, Murphy BR. Production of infectious human respiratory syncytial virus from cloned cDNA confirms an essential role for the transcription elongation factor from the 5' proximal open reading frame of the M2 mRNA in gene expression and provides a capability for vaccine development. *Proc Natl Acad Sci USA.* 1995; 92:11563–11567. [PubMed: 8524804]
- Collins PL, Hill MG, Cristina J, Grosfeld H. Transcription elongation factor of respiratory syncytial virus, a nonsegmented negative-strand RNA virus. *Proc Natl Acad Sci U S A.* 1996; 93(1):81–85. [PubMed: 8552680]
- Collins PL, Camargo E, Hill MG. Support plasmids and support proteins required for recovery of recombinant respiratory syncytial virus. *Virology.* 1999; 259(2):251–255. [PubMed: 10388648]
- Connors M, Kulkarni AB, Firestone CY, Holmes KL, Morse HCd, Sotnikov AV, Murphy BR. Pulmonary histopathology induced by respiratory syncytial virus (RSV) challenge of formalin-inactivated RSV-immunized BALB/c mice is abrogated by depletion of CD4 + T cells. *J Virol.* 1992; 66(12):7444–7451. [PubMed: 1433525]

- Cowton VM, Fearn R. Evidence that the respiratory syncytial virus polymerase is recruited to nucleotides 1 to 11 at the 3' end of the nucleocapsid and can scan to access internal signals. *J Virol.* 2005; 79(17):11311–11322. 79/17/11311 [pii]. 10.1128/JVI.79.17.11311-11322.2005 [PubMed: 16103183]
- Cowton VM, McGivern DR, Fearn R. Unravelling the complexities of respiratory syncytial virus RNA synthesis. *J Gen Virol.* 2006; 87(Pt 7):1805–1821. 87/7/1805 [pii]. 10.1099/vir.0.81786-0 [PubMed: 16760383]
- De Swart RL, Kuiken T, Timmerman HH, Amerongen GvG, Van Den Hoogen BG, Vos HW, Neijens HJ, Andeweg AC, Osterhaus AD. Immunization of macaques with formalin-inactivated respiratory syncytial virus (RSV) induces interleukin-13-associated hypersensitivity to subsequent RSV infection. *J Virol.* 2002; 76(22):11561–11569. [PubMed: 12388717]
- Drexler JF, Corman VM, Muller MA, Maganga GD, Vallo P, Binger T, Gloza-Rausch F, Rasche A, Yordanov S, Seebens A, Oppong S, Sarkodie YA, Pongombo C, Lukashev AN, Schmidt-Chanasit J, Stocker A, Carneiro AJ, Erbar S, Maisner A, Fronhoffs F, Buettner R, Kalko EK, Kruppa T, Franke CR, Kallies R, Yandoko ER, Herrler G, Reusken C, Hassanin A, Kruger DH, Matthee S, Ulrich RG, Leroy EM, Drosten C. Bats host major mammalian paramyxoviruses. *Nat Commun.* 2012; 3:796.10.1038/ncomms1796 [PubMed: 22531181]
- Dupuy LC, Dobson S, Bitko V, Barik S. Casein kinase 2-mediated phosphorylation of respiratory syncytial virus phosphoprotein P is essential for the transcription elongation activity of the viral polymerase; phosphorylation by casein kinase 1 occurs mainly at Ser(215) and is without effect. *J Virol.* 1999; 73(10):8384–8392. [PubMed: 10482589]
- Dyer KD, Percopo CM, Fischer ER, Gabryszewski SJ, Rosenberg HF. Pneumoviruses infect eosinophils and elicit MyD88-dependent release of chemoattractant cytokines and interleukin-6. *Blood.* 2009; 114(13):2649–2656.10.1182/blood-2009-01-199497 [PubMed: 19652202]
- Eisenhut M. Extrapulmonary manifestations of severe respiratory syncytial virus infection—a systematic review. *Crit Care.* 2006; 10(4):R107. cc4984 [pii]. 10.1186/cc4984 [PubMed: 16859512]
- Eshaghi A, Duvvuri VR, Lai R, Nadarajah JT, Li A, Patel SN, Low DE, Gubbay JB. Genetic variability of human respiratory syncytial virus strains circulating in Ontario: a novel genotype with a 72 nucleotide G gene duplication. *PLoS ONE.* 2012; 7(3):e32807.10.1371/journal.pone.0032807 [PubMed: 22470426]
- Fearn R, Collins PL. Model for polymerase access to the overlapped L gene of respiratory syncytial virus. *J Virol.* 1999a; 73(1):388–397. [PubMed: 9847343]
- Fearn R, Collins PL. Role of the M2-1 transcription antitermination protein of respiratory syncytial virus in sequential transcription. *J Virol.* 1999b; 73(7):5852–5864. [PubMed: 10364337]
- Fearn R, Peeples ME, Collins PL. Increased expression of the N protein of respiratory syncytial virus stimulates minigenome replication but does not alter the balance between the synthesis of mRNA and antigenome. *Virology.* 1997; 236(1):188–201. [PubMed: 9299631]
- Fearn R, Collins PL, Peeples ME. Functional analysis of the genomic and antigenomic promoters of human respiratory syncytial virus. *J Virol.* 2000; 74(13):6006–6014. [PubMed: 10846082]
- Fearn R, Peeples ME, Collins PL. Mapping the transcription and replication promoters of respiratory syncytial virus. *J Virol.* 2002; 76(4):1663–1672. [PubMed: 11799161]
- Fix J, Galloux M, Blondot ML, Eleouet JF. The insertion of fluorescent proteins in a variable region of respiratory syncytial virus L polymerase results in fluorescent and functional enzymes but with reduced activities. *Open Virol J.* 2011; 5:103–108.10.2174/1874357901105010103 [PubMed: 21966341]
- Fricke J, Koo LY, Brown CR, Collins PL. P38 and OGT sequestration into viral inclusion bodies in cells infected with human respiratory syncytial virus suppresses MK2 activities and stress granule assembly. *J Virol.* 2013; 87(3):1333–1347. [PubMed: 23152511]
- Fuentes S, Tran KC, Luthra P, Teng MN, He B. Function of the respiratory syncytial virus small hydrophobic protein. *J Virol.* 2007; 81(15):8361–8366. JVI.02717-06 [pii]. 10.1128/JVI.02717-06 [PubMed: 17494063]
- Fulginiti VA, Eller JJ, Sieber OF, Joyner JW, Minamitani M, Meiklejohn G. Respiratory virus immunization. I. A field trial of two inactivated respiratory virus vaccines; an aqueous trivalent

- parainfluenza virus vaccine and an alum-precipitated respiratory syncytial virus vaccine. *Am J Epidemiol.* 1969; 89(4):435–448. [PubMed: 4305199]
- Galloux M, Tarus B, Blazevic I, Fix J, Duquerroy S, Eleouet JF. Characterization of a viral phosphoprotein binding site on the surface of the respiratory syncytial nucleoprotein. *J Virol.* 2012; 86(16):8375–8387.10.1128/JVI.00058-12 [PubMed: 22623798]
- Gan SW, Tan E, Lin X, Yu D, Wang J, Tan GM, Vararattanavech A, Yeo CY, Soon CH, Soong TW, Pervushin K, Torres J. The small hydrophobic protein of the human respiratory syncytial virus forms pentameric ion channels. *J Biol Chem.* 2012; 287(29):24671–24689.10.1074/jbc.M111.332791 [PubMed: 22621926]
- Gershwin LJ, Schelegle ES, Gunther RA, Anderson ML, Woolums AR, Larochelle DR, Boyle GA, Friebertshauser KE, Singer RS. A bovine model of vaccine enhanced respiratory syncytial virus pathophysiology [In Process Citation]. *Vaccine.* 1998; 16(11–12):1225–1236. [PubMed: 9682383]
- Ghildyal R, Ho A, Jans DA. Central role of the respiratory syncytial virus matrix protein in infection. *FEMS Microbiol Rev.* 2006; 30(5):692–705.10.1111/j.1574-6976.2006.00025.x [PubMed: 16911040]
- Gonzalez-Reyes L, Ruiz-Arguello MB, Garcia-Barreno B, Calder L, Lopez JA, Albar JP, Skehel JJ, Wiley DC, Melero JA. Cleavage of the human respiratory syncytial virus fusion protein at two distinct sites is required for activation of membrane fusion. *Proc Natl Acad Sci U S A.* 2001; 98(17):9859–9864. [PubMed: 11493675]
- Gould PS, Easton AJ. Coupled translation of the second open reading frame of M2 mRNA is sequence dependent and differs significantly within the subfamily Pneumovirinae. *J Virol.* 2007; 81(16): 8488–8496. JVI.00457-07 [pii]. 10.1128/JVI.00457-07 [PubMed: 17522208]
- Graham BS, Henderson GS, Tang YW, Lu X, Neuzil KM, Colley DG. Priming immunization determines T helper cytokine mRNA expression patterns in lungs of mice challenged with respiratory syncytial virus. *J Immunol.* 1993; 151(4):2032–2040. [PubMed: 8345194]
- Grosfeld H, Hill MG, Collins PL. RNA replication by respiratory syncytial virus (RSV) is directed by the N, P, and L proteins; transcription also occurs under these conditions but requires RSV superinfection for efficient synthesis of full-length mRNA. *J Virol.* 1995; 69(9):5677–5686. [PubMed: 7637014]
- Groskreutz DJ, Babor EC, Monick MM, Varga SM, Hunninghake GW. Respiratory syncytial virus limits alpha subunit of eukaryotic translation initiation factor 2 (eIF2alpha) phosphorylation to maintain translation and viral replication. *J Biol Chem.* 2010; 285(31):24023–24031. M109.077321 [pii]. 10.1074/jbc.M109.077321 [PubMed: 20519500]
- Hanley LL, McGivern DR, Teng MN, Djang R, Collins PL, Fearn R. Roles of the respiratory syncytial virus trailer region: effects of mutations on genome production and stress granule formation. *Virology.* 2010; 406:241–252. S0042-6822(10)00446-0 [pii]. 10.1016/j.virol.2010.07.006 [PubMed: 20701943]
- Hardy RW, Wertz GW. The product of the respiratory syncytial virus M2 gene ORF1 enhances readthrough of intergenic junctions during viral transcription. *J Virol.* 1998; 72(1):520–526. [PubMed: 9420254]
- Hardy RW, Wertz GW. The Cys(3)-His(1) motif of the respiratory syncytial virus M2-1 protein is essential for protein function. *J Virol.* 2000; 74(13):5880–5885. [PubMed: 10846068]
- Harmon SB, Wertz GW. Transcriptional termination modulated by nucleotides outside the characterized gene end sequence of respiratory syncytial virus. *Virology.* 2002; 300(2):304–315. [PubMed: 12350361]
- Harpen M, Barik T, Musiyenko A, Barik S. Mutational analysis reveals a noncontractile but interactive role of actin and profilin in viral RNA-dependent RNA synthesis. *J Virol.* 2009; 83(21):10869–10876.10.1128/JVI.01271-09 [PubMed: 19710142]
- Haynes LM, Moore DD, Kurt-Jones EA, Finberg RW, Anderson LJ, Tripp RA. Involvement of toll-like receptor 4 in innate immunity to respiratory syncytial virus. *J Virol.* 2001; 75(22):10730–10737.10.1128/JVI.75.22.10730-10737.2001 [PubMed: 11602714]
- Henderson G, Murray J, Yeo RP. Sorting of the respiratory syncytial virus matrix protein into detergent-resistant structures is dependent on cell-surface expression of the glycoproteins. *Virology.* 2002; 300(2):244–254. [PubMed: 12350355]

- Hendricks DA, McIntosh K, Patterson JL. Further characterization of the soluble form of the G glycoprotein of respiratory syncytial virus. *J Virol.* 1988; 62(7):2228–2233. [PubMed: 3373568]
- Jeffree CE, Rixon HW, Brown G, Aitken J, Sugrue RJ. Distribution of the attachment (G) glycoprotein and GM1 within the envelope of mature respiratory syncytial virus filaments revealed using field emission scanning electron microscopy. *Virology.* 2003; 306(2):254–267. [PubMed: 12642099]
- Johnson RA, Prince GA, Suffin SC, Horswood RL, Chanock RM. Respiratory syncytial virus infection in cyclophosphamide-treated cotton rats. *Infect Immun.* 1982; 37(1):369–373. [PubMed: 7049952]
- Johnson JE, Gonzales RA, Olson SJ, Wright PF, Graham BS. The histopathology of fatal untreated human respiratory syncytial virus infection. *Mod Pathol.* 2007; 20(1):108–119. 3800725 [pii]. 10.1038/modpathol.3800725 [PubMed: 17143259]
- Johnson TR, Johnson CN, Corbett KS, Edwards GC, Graham BS. Primary human mDC1, mDC2, and pDC dendritic cells are differentially infected and activated by respiratory syncytial virus. *PLoS ONE.* 2011; 6(1):e16458.10.1371/journal.pone.0016458 [PubMed: 21297989]
- Johnson TR, McLellan JS, Graham BS. Respiratory syncytial virus glycoprotein G interacts with DC-SIGN and L-SIGN to activate ERK1 and ERK2. *J Virol.* 2012; 86(3):1339–1347.10.1128/JVI.06096-11 [PubMed: 22090124]
- Kakuk TJ, Soike K, Brideau RJ, Zaya RM, Cole SL, Zhang JY, Roberts ED, Wells PA, Wathen MW. A human respiratory syncytial virus (RSV) primate model of enhanced pulmonary pathology induced with a formalin-inactivated RSV vaccine but not a recombinant FG subunit vaccine. *J Infect Dis.* 1993; 167(3):553–561. [PubMed: 8440926]
- Kalica AR, Wright PF, Hetrick FM, Chanock RM. Electron microscopic studies of respiratory syncytial temperature-sensitive mutants. *Arch Gesamte Virusforsch.* 1973; 41(3):248–258. [PubMed: 4353798]
- Kapikian AZ, Mitchell RH, Chanock RM, Shvedoff RA, Stewart CE. An epidemiologic study of altered clinical reactivity to respiratory syncytial (RS) virus infection in children previously vaccinated with an inactivated RS virus vaccine. *Am J Epidemiol.* 1969; 89(4):405–421. [PubMed: 4305197]
- Karron RA, Buonagurio DA, Georgiu AF, Whitehead SS, Adamus JE, Clements-Mann ML, Harris DO, Randolph VB, Udem SA, Murphy BR, Sidhu MS. Respiratory syncytial virus (RSV) SH and G proteins are not essential for viral replication in vitro: clinical evaluation and molecular characterization of a cold-passaged, attenuated RSV subgroup B mutant. *Proc Natl Acad Sci U S A.* 1997a; 94(25):13961–13966. [PubMed: 9391135]
- Karron RA, Wright PF, Crowe JE Jr, Clements ML, Thompson J, Makhene M, Casey R, Murphy BR. Evaluation of two live, cold-passaged, temperature-sensitive respiratory syncytial virus (RSV) vaccines in chimpanzees, adults, infants and children. *J Infect Dis.* 1997b; 176:1428–1436. [PubMed: 9395351]
- Kim HW, Canchola JG, Brandt CD, Pyles G, Chanock RM, Jensen K, Parrott RH. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. *Am J Epidemiol.* 1969; 89(4):422–434. [PubMed: 4305198]
- Kim HW, Leikin SL, Arrobio J, Brandt CD, Chanock RM, Parrott RH. Cell-mediated immunity to respiratory syncytial virus induced by inactivated vaccine or by infection. *Pediatr Res.* 1976; 10(1):75–78. [PubMed: 1246465]
- Kolokoltsov AA, Deniger D, Fleming EH, Roberts NJ Jr, Karpilow JM, Davey RA. Small interfering RNA profiling reveals key role of clathrin-mediated endocytosis and early endosome formation for infection by respiratory syncytial virus. *J Virol.* 2007; 81(14):7786–7800. JVI.02780-06 [pii]. 10.1128/JVI.02780-06 [PubMed: 17494077]
- Kuo L, Grosfeld H, Cristina J, Hill MG, Collins PL. Effect of mutations in the gene-start and gene-end sequence motifs on transcription of monocistronic and dicistronic minigenomes of respiratory syncytial virus. *J Virol.* 1996; 70(10):6892–6901. [PubMed: 8794332]
- Kuo L, Fearn R, Collins PL. Analysis of the gene start and gene end signals of human respiratory syncytial virus: quasi-templated initiation at position 1 of the encoded mRNA. *J Virol.* 1997; 71(7):4944–4953. [PubMed: 9188557]
- Kwilas S, Liesman RM, Zhang L, Walsh E, Pickles RJ, Peeples ME. Respiratory syncytial virus grown in Vero cells contains a truncated attachment protein that alters its infectivity and dependence on

- glycosaminoglycans. *J Virol.* 2009; 83(20):10710–10718. JVI.00986-09 [pii]. 10.1128/JVI.00986-09 [PubMed: 19656891]
- Kwilas AR, Yednak MA, Zhang L, Liesman R, Collins PL, Pickles RJ, Peeples ME. Respiratory syncytial virus engineered to express CFTR corrects the bioelectric phenotype of human cystic fibrosis airway epithelium in vitro. *J Virol.* 2010; 84:7770–7781. JVI.00346-10 [pii]. 10.1128/JVI.00346-10 [PubMed: 20504917]
- Langedijk JP, de Groot BL, Berendsen HJ, van Oirschot JT. Structural homology of the central conserved region of the attachment protein G of respiratory syncytial virus with the fourth subdomain of 55-kDa tumor necrosis factor receptor. *Virology.* 1998; 243(2):293–302. [PubMed: 9568029]
- Lennon RG, Isacson P, Rosales T, Elsea WR, Karzon DT, Winkelstein W Jr. Skin tests with measles and poliomyelitis vaccines in recipients of inactivated measles virus vaccine. Delayed dermal hypersensitivity. *JAMA.* 1967; 200(4):275–280. [PubMed: 6071465]
- Li D, Jans DA, Bardin PG, Meanger J, Mills J, Ghildyal R. Association of respiratory syncytial virus M protein with viral nucleocapsids is mediated by the M2-1 protein. *J Virol.* 2008; 82(17):8863–8870.10.1128/JVI.00343-08 [PubMed: 18579594]
- Lifland AW, Jung J, Alonas E, Zurla C, Crowe JE Jr, Santangelo PJ. Human respiratory syncytial virus nucleoprotein and inclusion bodies antagonize the innate immune response mediated by MDA5 and MAVS. *J Virol.* 2012; 86(15):8245–8258.10.1128/JVI.00215-12 [PubMed: 22623778]
- Lindquist ME, Lifland AW, Utley TJ, Santangelo PJ, Crowe JE Jr. Respiratory syncytial virus induces host RNA stress granules to facilitate viral replication. *J Virol.* 2010; 84(23):12274–12284.10.1128/JVI.00260-10 [PubMed: 20844027]
- Lindquist ME, Mainou BA, Dermody TS, Crowe JE Jr. Activation of protein kinase R is required for induction of stress granules by respiratory syncytial virus but dispensable for viral replication. *Virology.* 2011; 413(1):103–110.10.1016/j.virol.2011.02.009 [PubMed: 21377708]
- Liuzzi M, Mason SW, Cartier M, Lawetz C, McCollum RS, Dansereau N, Bolger G, Lapeyre N, Gaudette Y, Lagace L, Massariol MJ, Do F, Whitehead P, Lamarre L, Scouten E, Bordeleau J, Landry S, Rancourt J, Fazal G, Simoneau B. Inhibitors of respiratory syncytial virus replication target cotranscriptional mRNA guanylation by viral RNA-dependent RNA polymerase. *J Virol.* 2005; 79(20):13105–13115. [PubMed: 16189012]
- Llorente MT, Garcia-Barreno B, Calero M, Camafeita E, Lopez JA, Longhi S, Ferron F, Varela PF, Melero JA. Structural analysis of the human respiratory syncytial virus phosphoprotein: characterization of an alpha-helical domain involved in oligomerization. *J Gen Virol.* 2006; 87(Pt 1):159–169. 87/1/159 [pii]. 10.1099/vir.0.81430-0 [PubMed: 16361428]
- Lu B, Ma CH, Brazas R, Jin H. The major phosphorylation sites of the respiratory syncytial virus phosphoprotein are dispensable for virus replication in vitro. *J Virol.* 2002; 76(21):10776–10784. [PubMed: 12368320]
- Marsh R, Connor A, Gias E, Toms GL. Increased susceptibility of human respiratory syncytial virus to neutralization by anti-fusion protein antibodies on adaptation to replication in cell culture. *J Med Virol.* 2007; 79(6):829–837.10.1002/jmv.20892 [PubMed: 17457900]
- McCurdy LH, Graham BS. Role of plasma membrane lipid microdomains in respiratory syncytial virus filament formation. *J Virol.* 2003; 77(3):1747–1756. [PubMed: 12525608]
- McDonald TP, Pitt AR, Brown G, Rixon HW, Sugrue RJ. Evidence that the respiratory syncytial virus polymerase complex associates with lipid rafts in virus-infected cells: a proteomic analysis. *Virology.* 2004; 330(1):147–157. [PubMed: 15527841]
- McGivern DR, Collins PL, Fearn R. Identification of internal sequences in the 3' leader region of human respiratory syncytial virus that enhance transcription and confer replication processivity. *J Virol.* 2005; 79(4):2449–2460. [PubMed: 15681446]
- Mitra R, Baviskar P, Duncan-Decocq RR, Patel D, Oomens AG. The human respiratory syncytial virus matrix protein is required for maturation of viral filaments. *J Virol.* 2012; 86(8):4432–4443.10.1128/JVI.06744-11 [PubMed: 22318136]
- Money VA, McPhee HK, Mosely JA, Sanderson JM, Yeo RP. Surface features of a Mononegavirales 4441–4446. 0805740106 [pii]. 10.1073/pnas.0805740106 [PubMed: 19251668]

- Monsalvo AC, Bataille JP, Lopez MF, Krause JC, Klemenc J, Hernandez JZ, Maskin B, Bugna J, Rubinstein C, Aguilar L, Dalurzo L, Libster R, Savy V, Baumeister E, Aguilar L, Cabral G, Font J, Solari L, Weller KP, Johnson J, Echavarria M, Edwards KM, Chappell JD, Crowe JE Jr, Williams JV, Melendi GA, Polack FP. Severe pandemic 2009 H1N1 influenza disease due to pathogenic immune complexes. *Nat Med*. 2011; 17(2):195–199.10.1038/nm.2262 [PubMed: 21131958]
- Murphy BR, Walsh EE. Formalin-inactivated respiratory syncytial virus vaccine induces antibodies to the fusion glycoprotein that are deficient in fusion-inhibiting activity. *J Clin Microbiol*. 1988; 26(8):1595–1597. [PubMed: 2459154]
- Murphy BR, Prince GA, Walsh EE, Kim HW, Parrott RH, Hemming VG, Rodriguez WJ, Chanock RM. Dissociation between serum neutralizing and glycoprotein antibody responses of infants and children who received inactivated respiratory syncytial virus vaccine. *J Clin Microbiol*. 1986; 24(2):197–202. [PubMed: 3755730]
- Noton SL, Fearn R. The first two nucleotides of the respiratory syncytial virus antigenome RNA replication product can be selected independently of the promoter terminus. *RNA*. 2011; 17(10): 1895–1906.10.1261/rna.2813411 [PubMed: 21878549]
- Noton SL, Cowton VM, Zack CR, McGivern DR, Fearn R. Evidence that the polymerase of respiratory syncytial virus initiates RNA replication in a nontemplated fashion. *Proc Natl Acad Sci U S A*. 2010; 107(22):10226–10231. 0913065107 [pii]. 10.1073/pnas.0913065107 [PubMed: 20479224]
- Noton SL, DeFlube LR, Tremaglio CZ, Fearn R. The respiratory syncytial virus polymerase has multiple RNA synthesis activities at the promoter. *PLoS Path*. 2012; 8(10):e1002980.
- Peeples ME, Collins PL. Mutations in the 5' trailer region of a respiratory syncytial virus minigenome which limit RNA replication to one step. *J Virol*. 2000; 74(1):146–155. [PubMed: 10590101]
- Polack FP, Teng MN, Collins PL, Prince GA, Exner M, Regele H, Lirman DD, Rabold R, Hoffman SJ, Karp CL, Kleeberger SR, Wills-Karp M, Karron RA. A role for immune complexes in enhanced respiratory syncytial virus disease. *J Exp Med*. 2002; 196(6):859–865. [PubMed: 12235218]
- Polack FP, Irusta PM, Hoffman SJ, Schiatti MP, Melendi GA, Delgado MF, Laham FR, Thumar B, Hendry RM, Melero JA, Karron RA, Collins PL, Kleeberger SR. The cysteine-rich region of respiratory syncytial virus attachment protein inhibits innate immunity elicited by the virus and endotoxin. *Proc Natl Acad Sci U S A*. 2005; 102(25):8996–9001. [PubMed: 15956195]
- Rawling J, Cano O, Garcin D, Kolakofsky D, Melero JA. Recombinant Sendai viruses expressing fusion proteins with two furin cleavage sites mimic the syncytial and receptor-independent infection properties of respiratory syncytial virus. *J Virol*. 2011; 85(6):2771–2780. JVI.02065-10 [pii]. 10.1128/JVI.02065-10 [PubMed: 21228237]
- Rezaee F, Gibson LF, Piktel D, Othumpangat S, Piedimonte G. Respiratory syncytial virus infection in human bone marrow stromal cells. *Am J Respir Cell Mol Biol*. 2011; 45(2):277–286.10.1165/rcmb.2010-0121OC [PubMed: 20971883]
- Roberts SR, Compans RW, Wertz GW. Respiratory syncytial virus matures at the apical surfaces of polarized epithelial cells. *J Virol*. 1995; 69(4):2667–2673. [PubMed: 7884920]
- Rohwedder A, Keminer O, Forster J, Schneider K, Schneider E, Werchau H. Detection of respiratory syncytial virus RNA in blood of neonates by polymerase chain reaction. *J Med Virol*. 1998; 54(4):320–327. [PubMed: 9557299]
- Sastre P, Oomens AG, Wertz GW. The stability of human respiratory syncytial virus is enhanced by incorporation of the baculovirus GP64 protein. *Vaccine*. 2007; 25(27):5025–5033. S0264-410X(07)00506-3 [pii]. 10.1016/j.vaccine.2007.04.066 [PubMed: 17544182]
- Spann KM, Collins PL, Teng MN. Genetic recombination during coinfection of two mutants of human respiratory syncytial virus. *J Virol*. 2003; 77(20):11201–11211. [PubMed: 14512568]
- Spann KM, Tran KC, Collins PL. Effects of nonstructural proteins NS1 and NS2 of human respiratory syncytial virus on interferon regulatory factor 3, NF-kappaB, and proinflammatory cytokines. *J Virol*. 2005; 79(9):5353–5362. [PubMed: 15827150]
- Srinivasakumar N, Ogra PL, Flanagan TD. Characteristics of fusion of respiratory syncytial virus with HEp-2 cells as measured by R18 fluorescence dequenching assay. *J Virol*. 1991; 65(8):4063–4069. [PubMed: 1906550]

- Surman SR, Collins PL, Murphy BR, Skiadopoulos MH. An improved method for the recovery of recombinant paramyxovirus vaccine candidates suitable for use in human clinical trials. *J Virol Methods*. 2007; 141(1):30–33. S0166-0934(06)00409-5 [pii]. 10.1016/j.jviromet.2006.11.024 [PubMed: 17210187]
- Swedan S, Andrews J, Majumdar T, Musiyenko A, Barik S. Multiple functional domains and complexes of the two nonstructural proteins of human respiratory syncytial virus contribute to interferon suppression and cellular location. *J Virol*. 2011; 85(19):10090–10100.10.1128/JVI.00413-11 [PubMed: 21795342]
- Tawar RG, Duquerroy S, Vonnrhein C, Varela PF, Damier-Piolle L, Castagne N, MacLellan K, Bedouelle H, Bricogne G, Bhella D, Eleouet JF, Rey FA. Crystal structure of a nucleocapsid-like nucleoprotein-RNA complex of respiratory syncytial virus. *Science*. 2009; 326(5957):1279–1283. 326/5957/1279 [pii]. 10.1126/science.1177634 [PubMed: 19965480]
- Tayyari F, Marchant D, Moraes TJ, Duan W, Mastrangelo P, Hegele RG. Identification of nucleolin as a cellular receptor for human respiratory syncytial virus. *Nat Med*. 2011; 17:1132–1135. [PubMed: 21841784]
- Teng MN, Collins PL. Identification of the respiratory syncytial virus proteins required for formation and passage of helper-dependent infectious particles. *J Virol*. 1998; 72(7):5707–5716. [PubMed: 9621029]
- Teng MN, Collins PL. The central conserved cystine noose of the attachment G protein of human respiratory syncytial virus is not required for efficient viral infection in vitro or in vivo. *J Virol*. 2002; 76(12):6164–6171. [PubMed: 12021350]
- Teng MN, Whitehead SS, Bermingham A, Clair MS, Elkins WR, Murphy BR, Collins PL. Recombinant respiratory syncytial virus that does not express the NS1 or M2-2 protein is highly attenuated and immunogenic in chimpanzees. *J Virol*. 2000; 74:9317–9321. [PubMed: 10982380]
- Tran TL, Castagne N, Dubosclard V, Noinville S, Koch E, Moudjou M, Henry C, Bernard J, Yeo RP, Eleouet JF. The respiratory syncytial virus M2-1 protein forms tetramers and interacts with RNA and P in a competitive manner. *J Virol*. 2009; 83(13):6363–6374. JVI.00335-09 [pii]. 10.1128/JVI.00335-09 [PubMed: 19386701]
- Tripp RA, Jones LP, Haynes LM, Zheng H, Murphy PM, Anderson LJ. CX3C chemokine mimicry by respiratory syncytial virus G glycoprotein. *Nat Immunol*. 2001; 2(8):732–738. [PubMed: 11477410]
- Uitley TJ, Ducharme NA, Varthakavi V, Shepherd BE, Santangelo PJ, Lindquist ME, Goldenring JR, Crowe JE Jr. Respiratory syncytial virus uses a Vps4-independent budding mechanism controlled by Rab11-FIP2. *Proc Natl Acad Sci U S A*. 2008; 105(29):10209–10214. 0712144105 [pii]. 10.1073/pnas.0712144105 [PubMed: 18621683]
- Venter M, van Niekerk S, Rakgantso A, Bent N. Identification of deletion mutant respiratory syncytial virus strains lacking most of the G protein in immunocompromised children with pneumonia in South Africa. *J Virol*. 2011; 85(16):8453–8457.10.1128/JVI.00674-11 [PubMed: 21680500]
- Villanave R, Thavagnanam S, Sarlang S, Parker J, Douglas I, Skibinski G, Heaney LG, McKaigue JP, Coyle PV, Shields MD, Power UF. In vitro modeling of respiratory syncytial virus infection of pediatric bronchial epithelium, the primary target of infection in vivo. *Proc Natl Acad Sci U S A*. 2012; 109(13):5040–5045.10.1073/pnas.1110203109 [PubMed: 22411804]
- Wang JT, McElvain LE, Whelan SP. Vesicular stomatitis virus mRNA capping machinery requires specific cis-acting signals in the RNA. *J Virol*. 2007; 81(20):11499–11506.10.1128/JVI.01057-07 [PubMed: 17686869]
- Whitehead SS, Juhasz K, Firestone CY, Collins PL, Murphy BR. Recombinant respiratory syncytial virus (RSV) bearing a set of mutations from cold-passaged RSV is attenuated in chimpanzees. *J Virol*. 1998; 72(5):4467–4471. [PubMed: 9557743]
- Whitehead SS, Bukreyev A, Teng MN, Firestone CY, St Claire M, Elkins WR, Collins PL, Murphy BR. Recombinant respiratory syncytial virus bearing a deletion of either the NS2 or SH gene is attenuated in chimpanzees. *J Virol*. 1999; 73(4):3438–3442. [PubMed: 10074199]
- Wright PF, Ikizler MR, Gonzales RA, Carroll KN, Johnson JE, Werkhaven JA. Growth of respiratory syncytial virus in primary epithelial cells from the human respiratory tract. *J Virol*. 2005; 79(13):8651–8654. 79/13/8651 [pii]. 10.1128/JVI.79.13.8651-8654.2005 [PubMed: 15956607]

- Wright PF, Karron RA, Belshe RB, Shi JR, Randolph VB, Collins PL, O'Shea AF, Gruber WC, Murphy BR. The absence of enhanced disease with wild type respiratory syncytial virus infection occurring after receipt of live, attenuated, respiratory syncytial virus vaccines. *Vaccine*. 2007; 25(42):7372–7378. S0264-410X(07)00937-1 [pii]. 10.1016/j.vaccine.2007.08.014 [PubMed: 17868959]
- Yeo DS, Chan R, Brown G, Ying L, Sutejo R, Aitken J, Tan BH, Wenk MR, Sugrue RJ. Evidence that selective changes in the lipid composition of raft-membranes occur during respiratory syncytial virus infection. *Virology*. 2009; 386(1):168–182. S0042-6822(08)00799-X [pii]. 10.1016/j.virol.2008.12.017 [PubMed: 19178924]
- Zhang L, Peeples ME, Boucher RC, Collins PL, Pickles RJ. Respiratory syncytial virus infection of human airway epithelial cells is polarized, specific to ciliated cells, and without obvious cytopathology. *J Virol*. 2002; 76(11):5654–5666. [PubMed: 11991994]

Fig. 1.

Photomicrographs (**a** and **b**) and electron micrographs (**c–e**) of RSV-infected cells and associated viral structures. **a** is a photomicrograph of a syncytium in an RSV-infected cell monolayer (several nuclei are indicated with *arrows*; courtesy of Dr. Alexander Bukreyev). **b** is a fluorescence photomicrograph of a syncytium in an RSV-infected cell monolayer (not the same one as in **a**) stained with an antibody specific to the F protein, showing filamentous viral projections (courtesy of Dr. Ursula J. Buchholz). **c** is an electron micrograph of negatively stained budding RSV virions: V indicates a budding virion and F indicates filamentous cytoplasmic structures that likely are nucleocapsids (courtesy of Dr. Robert M. Chanock) (Kalica et al. 1973). **d** and **e** are field emission scanning electron micrographs of the surface of uninfected (**d**) and RSV-infected (**e**) cells, illustrating viral filamentous structures (VF in **e**) that are thought to form at sites of virus budding and may yield filamentous particles; also shown are microvilli (mv in **d**) that are found in uninfected cells (courtesy of Dr. Richard Sugrue) (Jeffree et al. 2003)

Fig. 2. RSV proteins and their functions and location in the virion, shown in reference strained electron micrographs of negatively stained budding (a) and free (b) virions (courtesy of Dr. Robert M. Chanock)

Fig. 3.

Diagram of the 3' to 5' negative-sense RSV genome (approximately to scale, strain A2), and sequences of the leader region, gene junctions, overlap, and trailer region. **a** Genome diagram: each *box* represents a gene encoding a separate mRNA. The *first row* of numbers over the diagram indicates the nucleotide lengths of the genes, and the second, *upper row* of numbers (*italicized*) indicates the amino acid lengths of the primary, unmodified proteins. The overlapping ORFs of the M2 mRNA are illustrated over the M2 gene. The numbers under the diagram indicate the lengths of the leader, intergenic, and trailer regions (*underlined*) and the gene overlap (*parentheses*). **b** Sequences of the leader region, gene junctions, overlap, and trailer region (3' to 5', negative-sense). This shows the leader region, followed by the NS1, NS2, N, P, M, SH, G, and F genes and their intergenic regions, followed by the overlapping M2 and L genes and the trailer region. The main body of each gene is deleted and is represented by a *box* with the gene name. Nucleotide assignments that are conserved between the 3' ends of the genome and the antigenome (represented here as the reverse complement in the trailer region) are in **bold capitals**. The gene-start and gene-end signals of the gene are underlined, and conserved assignments are in *capitals*

Fig. 4.

Overview of RSV transcription and RNA replication. The polymerase enters the negative-sense genome at its 3' end executes transcription to yield positive-sense subgenomic mRNAs (in a polar gradient) or executes the first step in RNA replication to yield full-length positive-sense antigenome. The polymerase enters the antigenome at its 3' end and executes the second step of RNA replication to yield full-length progeny genomes. Note that the L gene yields two polyadenylated mRNAs: a very short species due to termination in the gene overlap, and full-length L mRNA

Fig. 5.

Comparison of the genes and gene order of RSV with those of selected members of *Paramyxoviridae*: HMPV, human parainfluenza virus (HPIV) serotypes 1, 2, and 3, and measles virus (MeV). The genes are shown in their 3' to 5' order in genomic RNA, which is the direction of transcription. Genes are not to scale, and orthologous genes are aligned vertically as much as possible (the only genes that could not be appropriately aligned are SH and G of RSV and SH of HMPV), with gaps introduced to maximize the alignments. Genes encoding major protective antigens are in *dark shading*. Asterisks indicate proteins that can be deleted from RSV without loss of replication, although this may be reduced. Proteins that have no direct ortholog in RSV include: *C* small accessory protein, *V* cysteine-rich accessory protein, *HN* hemagglutinin-neuraminidase glycoprotein, *H* hemagglutinin glycoprotein. The *Henipavirus* and *Avulavirus* genera and a number of unclassified viruses within *Paramyxovirinae* are not represented

Fig. 6.

Nucleotide positions in the leader region of genomic RNA that are important for transcription (*top*) and RNA replication (*bottom*). Important residues present in positions 1–11 are indicated with *open boxes*; note that those that are important for transcription are a subset of those important for RNA replication. A region that increases the efficiency of transcription is indicated with a *dashed box*. The GS signal of the first gene, necessary for transcriptional initiation but not involved in RNA replication, is *underlined* in the diagram for transcription. A region that contains an apparent encapsidation signal necessary to produce full-length replication products is indicated with a *shaded box*. Sequences are in negative-sense

Fig. 7.

Reverse genetic systems. **A** Helper-dependent mini-replicon system, illustrated with a dicistronic mini-replicon (shown here as an antigenome-sense replicon) containing chloramphenicol acetyl transferase (CAT) and luciferase (LUC) marker genes under the control of RSV GS and GE signals (not shown) and flanked by the complements of the RSV leader and trailer regions (*gray boxes*, *lec* and *trc*, respectively). The mini-replicon cDNA is flanked by a T7 RNA polymerase promoter (*arrow*, T7 pr) and a self-cleaving ribozyme (*black box*, *rbz*). This is complemented by support plasmids encoding various RSV proteins. **b** Recovery of complete infectious virus from a full-length antigenome expressed from a transfected plasmid in the presence of support plasmids encoding the N, P, M2-1, and L

proteins. The complements of the leader and trailer regions are shown (*gray boxes*, *lec* and *trc*, respectively), as are the T7 RNA polymerase promoter (*arrow*, *T7pr*) and ribozyme (*black box*)

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Amino acid sequence identity between the proteins of RSV subgroup A (RSV-A) and the indicated members of subfamily *Pneumovirinae*^a

Table 1

Viruses compared	Amino acid sequence identity for the indicated protein (%)										
	NS1	NS2	N	P	M	SH	G	F	M2-1	M2-2	L
RSV-A versus RSV-B	87	92	96	91	91	76	53	89	92	72	93
BRSV	69	84	93	81	89	38	30	81	80	42	84
PVM	16	20	60	33	42	23	12	43	43	10	53
HMPV-A	<i>b</i>	<i>b</i>	42	35	38	23	15	33	36	17	45
AMPV-A	<i>b</i>	<i>b</i>	41	32	38	19	16	35	37	12	43

^a Viruses are listed in order of decreasing relatedness to HRSV-A. RSV-B is RSV subgroup B; HMPV-A and AMPV-A are subgroup A of human and avian human metapneumovirus. Viruses in *Parainflavirinae* are not shown for comparison because the percent identity is <20 % at the level of the entire protein

^b This virus does not have this gene

Table 2

Notable features of RSV

Replication and budding in vitro are inefficient, infectivity is unstable, particles grown in vitro are mostly large filaments
RSV encodes additional proteins that are either unique to the genus (NS1 and NS2) or found only in a subset of viruses in <i>Paramyxoviridae</i> (SH, M2-1, and M2-2)
Two genes, NS1 and NS2, are dedicated to expressing proteins that interfere with the host type I interferon system, among other functions
Overlapping ORFs in the M2 mRNA encode factors that confer transcription processivity (M2-1) or shift viral RNA synthesis from transcription to RNA replication (M2-2)
The M2 and L genes overlap and L mRNA is expressed by a backtracking mechanism
The small hydrophobic (SH) protein forms a pentameric ion channel, but its function is unclear
The F protein precursor is activated by cleavage at two furin recognition sites
The F protein activates TLR-4 signaling pathways, but this is inhibited by the G protein
Viral attachment appears to involve both the F and G proteins, but F fuses independently of G
The G protein is heavily glycosylated, nonglobular, and highly variable
The G protein bears a CX3C fractalkine-like motif that may modify the cellular immune response
The G protein is expressed in membrane-bound and secreted forms; the latter interferes with antibody-mediated neutralization, and interacts directly with antigen presenting cells to modify their function

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